# Polyethylene oxide-Polyester Micelles for Drug Delivery

BY

David S. Hart

Submitted to the Department of Pharmaceutical Chemistry
and the Faculty of the Graduate School of the University of Kansas
In partial fulfillment of the requirements for the degree of
Master's of Science

Christian Schöneich
Chairperson of Department

Committee Members: M. Laird Forrest, PhD

Cory Berkland, PhD

Teruna J. Siahaan, PhD

Date defended: October 15, 2007

The Thesis Committee for David S. Hart certifies
That this is the approved Version of the following thesis:

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**Abstract**

Poly(ethylene oxide) (PEO)-poly(ester) copolymers are biocompatible, biodegradable diblock polymers that spontaneously form core-shell nanoparticles (i.e. micelles) in water. The hydrophobic polyester core of these micelles can solubilize small, hydrophobic drug molecules that have shown promise in the treatment of cancer and infectious diseases. This core has undergone much development in recent years to improve the stability, drug loading capacity, and release rates of drugs. The PEO shell is well known to increase the circulatory lifetime of micelles with little or no immune response. This thesis will emphasize PEO-poly(ε-caprolactone) micelles in order to highlight recent advances in this area.

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## 1.0 Introduction

Many of the > 1,000 natural products that have been approved by FDA in the last 25 years for the treatment of cancer and infectious diseases have very poor water solubility.[1] This problem continues today in many pharmaceutical companies, as high throughput combinatorial screening frequently identifies drug targets with low water solubility.[2-4] Traditional techniques to improve aqueous solubility, such as chemical modifications and pH adjustments to form salts from ionizable groups often reduce or destroy these drugs therapeutic activity. Thus unmodified drugs move on to become candidates and are so hydrophobic, they cannot be delivered via intravenous infusion, or without cytotoxic delivery vehicles such as Cremophor® EL, Tween, or harsh organic solvents such as ethanol and DMSO.[5] Therefore drug delivery vehicles that can solubilize and encapsulate high concentrations of these drugs are needed to formulate these prospective drug candidates. The polyester core of these micelles can encapsulate these hydrophobic drugs because alkane rich (e.g. pentanes in poly-ε-caprolactones) segments between these esters increase the thermodynamic affinity of the drugs to the core. Polyester cores are biodegradable through esterases *in vivo*. There are three main ester monomers commonly used as cores: ε-caprolactone, $_{D,L}$-Lactic acid, and $_{D,L}$-Lactic acid-co glycolic acid. Recently, there have been many excellent reviews on the drug delivery from micelles of PEO-poly($_{D,L}$-Lactic acid)[6-10] and PEO-poly($_{D,L}$-Lactic acid-co glycolic acid).[11-13] Therefore this review will focus on poly(ε-caprolactone) linear, star, and substituted

cores in order to highlight recent developments in this area. These include improving stability, loading capacity, and tailoring release rates.

The Poly(ethylene oxide) PEO portion of PEO-polyester micelles resides at the periphery of the micelle and is often referred to as the corona or shell. PEO has been deemed a "generally regarded as safe" molecule by the FDA, and has little or no immunogenicity. When the micelles are less than approximately 200 nm in diameter, uptake by the reticuloendothelial systems (RES) of the liver and spleen is limited. This lack of immune system recognition in conjunction with reduced RES uptake dramatically increases the circulatory lifetime of these micelles.[14, 15] In order for PEO- poly(ε-caprolactone) (PCL) micelles to be clinically effective, they need to have structural integrity *in vivo*, or low critical-micelle concentrations (CMC), and control the release of the target drugs. The molecular weights of the core and shell can be designed to optimize micelle size, CMC, encapsulation efficiency, and rate of drug release. Understanding the thermodynamic affinity of a drug molecule for the micelle core is paramount to the design of future drug delivery systems. This understanding will allow researchers to foresee problems with encapsulation, and attack those problems with substituted cores, additives to increase lipophilicity, or by simply adjusting the core block length. The synthesis of these micelles, encapsulation, and release characterizations of anticancer and immunosuppressive drugs, as well as drug models will be described. The assessment of the fate of these

micelles *in vivo*, cell internalization studies, pharmacokinetics of drugs and micelles, and tissue distribution studies will conclude this review.

## 1.1 Synthesis of PEO-poly(ε-caprolactone)

PEO-poly(ε-caprolactone) (PCL) is typically synthesized by a ring opening polymerization of ε-caprolactone from the terminal hydroxyl of PEO (Figure 1). Ring-opening polymerizations are generally catalyzed by Stannous (II) octoate,[16] or hydrochloric acid,[17] but can be non-catalytically polymerized at high temperatures (160-200°C) for several days.[18] Other catalysts such as antimony compounds suffer from toxicity,[19] whereas stannous octoate is FDA approved as a food additive, and HCl is commonly used in the formulation of pharmaceutics. The extent of polymerization is verified through comparison of characteristic peak shift intensities of PEO (3.39 and 3.64 ppm) and poly(ε-caprolactone) (1.38, 1.65, 2.31, and 4.06 ppm) through $H^1$-NMR in $CDCl_3$ (Figure 2).

This synthesis has become routine to the point that yields are often >90% with relatively monodisperse polymers (1.0-1.2). The preparation of PEO-PCL micelles in aqueous solution usually relies on polymer and drug in organic solvent being slowly added drop wise to aqueous solvent.[20-23] Sonication can also be used to solubilize PCL directly in aqueous solution.[24, 25] Dialysis followed by lyophilization or solvent evaporation is then used to remove free drug and organic solvent.[22, 26] Lyoprotectants, such as sugars can be used during freeze drying to ensure colloidal stability upon resuspension in aqueous solution.[27] Following purification and

drying, these polymers can be evaluated by a variety of conventional solid characterization techniques.

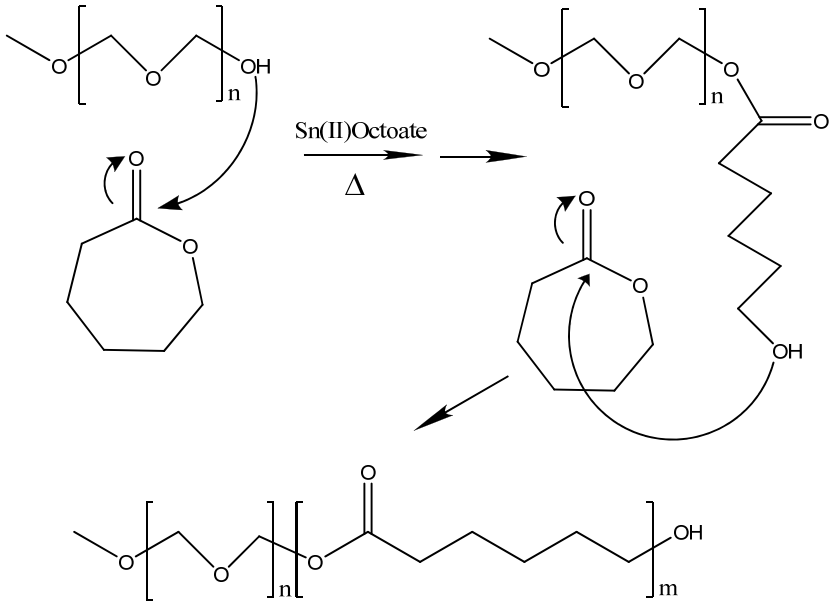


Figure 1. Catalyzed polymerization of PCL (m units) with methoxy-PEO (n units)

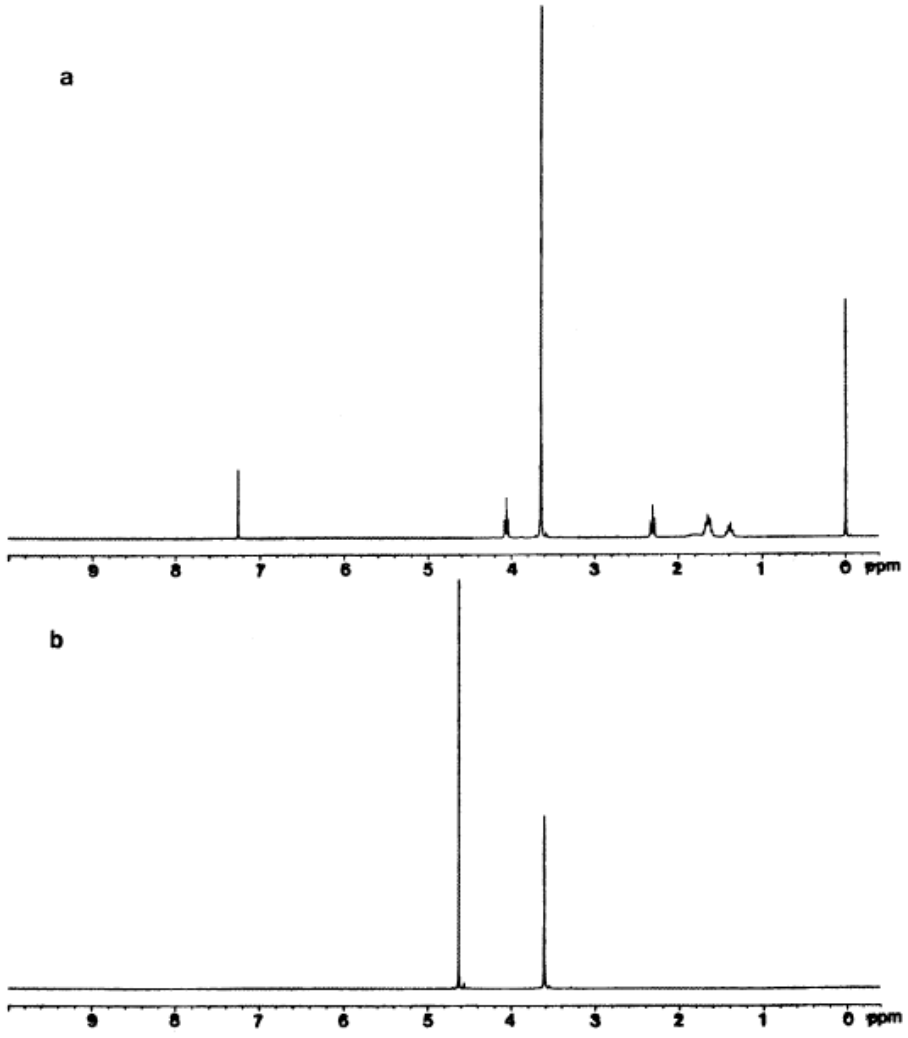


Figure 2. Representative $H^1$-NMR spectra of a PEO-PCL triblock copolymer in (a) $CDCl_3$ and (b) water. From reference [62].

## 1.2 Polymer Characterization of PEO-PCL

As PEO has been well characterized [28-30] as a polymer and in biological systems, the development of new PCL cores often warrants the copolymers characterization. PCL is a crystalline polymer, known to act as a rubber at room temperature since its glass transition temperature ($T_g$) is -60°C and its melting point

($T_m$) is 58.5°C when it is 21 kDa.[31] Thus its crystallinity is less affected through the addition of solutes into the PCL core of micelles compared to polylactic acid. Both the $T_g$ and $T_m$ are usually measured with DSC (differential scanning calorimetry). X-ray diffraction is also used to decipher the amorphorous or crystalline state of copolymers with or without drugs.[32-34] A variety of PEO-PCL co-polymers have been developed for drug delivery; from conventional diblocks and triblocks to more exotic micelles with multi-arm stars, and substituted PCL blocks. Therefore the following section will review the physical characterization of PEO-PCL micelles in aqueous solution.

## 1.3 Characterization of PEO-PCL micelles

In the context of micelle-based drug delivery, encapsulation efficiency and release are irrevocably related to the equilibrium between unimers and micelles in aqueous solution. This equilibrium can be inferred indirectly from CMC measurements. The aggregation number ($N_{agg}$) determined from small angle neutron scattering (SANS) or fluorescent dye quenching techniques [35] provides a number average of unimers required to form a micelle. Self-diffusion coefficients of free PEO in solution obtained through NMR provide a relative scale to access the presence of unimers in solution. This knowledge combined with the conformation and shape of the micelles provides a physical model of amphiphilic copolymer association on a thermodynamic and molecular basis. Understanding the discrete

association of PEO-PCL copolymers may allow future block copolymers to be designed which don't require harsh conditions (e.g. organic solvents, sonication, or heating and stirring) to form micelles in aqueous solution.

Jérôme and colleagues correlated SANS and small-angle x-ray scattering (SAXS) data with statistical mechanics models to more accurately describe the self association of PEO-PCL polymers in aqueous solution.[36] In order to dissolve the polymers directly in aqueous solution with heating a series of PEO-PCL polymers were synthesized with a constant PEO block length of 5 kDa, and relatively short PCL blocks (0.35, 0.95, 1.5, 1.85, 2.2, and 3 kDa). The conformation of the PEO chains in the corona were determined to exist in a moderately extended geometry based on steric hindrance at the corona-core interface depicted by its low surface area. The surface area of this interface was calculated based on $N_{agg}$. Aggregation numbers ranged from 12 to 126 for PCL block lengths of 0.35-2.2 kDa. SANS and SAXS data were fit to various models to determine the conformation of core, which was depicted as a dense core of rods, based on the size and model. Micelle size was determined to be 20-25 nm in diameter for PCL block lengths of 0.95-2.2 kDa. Jérôme and coworkers have also shown that when micelles are formed from organic solvent and water, lower concentrations of copolymers lead to larger micelles, and depending on the solvent, the size can range from 30-100 nm.[37] The CMCs determined from surface tension data were on the order of $10^{-5}$-$10^{-6}$ M for PEO-PCL micelles with PCL block lengths ranging from 0.95-2.2 kDa. Using self-diffusion coefficients obtained from NMR, a higher content of unimers were present with

smaller PCL blocks because their diffusion coefficients were very close to that of free PEO in solution. Transmission electron microscopy confirmed the spherical shape and monodispersity of the micelles.

Table 1. Influence of PCL block length, arrangement, and substitution on CMC

| Copolymer | Block arrangement/ geometry | PCL Core MW range (kDa) | PEO MW (kDa) | CMC range (M) | Ref. |
|---|---|---|---|---|---|
| PEO-PCL | diblock | 0.4-2.2 | 5.0 | $10^{-5}$-$10^{-6}$ | [36] |
| PEO-PVL | diblock | 0.5-2.0 | 2.0 | $10^{-5}$-$10^{-6}$ | [53] |
| PEO-PVL | diblock | 1.1-4.9 | 5.0 | $10^{-5}$-$10^{-6}$ | [53] |
| $(PEO)_6$-PVL | 6-arm star | 4.9-28.8 [b] | 11-22 [b] | $10^{-6}$ | [32] |
| PEO-PCCL | PCL-block w/ α-carboxylate | 1.8 | 5.0 | $10^{-6}$ | [68] |
| PEO-PCL | diblock | 2.5-4.0 | 5.0 | $10^{-7}$ | [22] |
| PEO-PCL | diblock | 10.0 | 5.0 | $10^{-7}$ | [60] |
| PCL-PEO-PCL | triblock | 4.8-7.5[a] | 8.0 | $10^{-7}$ | [62] |
| PEO-PCL | diblock | 18.0 | 5.0 | $10^{-8}$ | [60] |
| PEO-PCL | diblock | 13.0-24.0 | 5.0 | $10^{-8}$ | [45] |
| PCL-PEO-PCL | triblock | 15.8[a] | 8.0 | $10^{-8}$ | [62] |
| PEO-$(PCL)_3$ | 3-arm star | 14.2[b] | 14.8 | $10^{-8}$ | [66] |
| PEO-$(PCL)_4$ | 4-arm star | 20.4[b] | 5.2 | $10^{-8}$ | [66] |
| PEO-PBCL | PCL-block w/ α-benzyl carboxylate | 2.0 | 5.0 | $10^{-8}$ | [68] |

[a]This MW is the sum of each block
[b]This MW is the sum of each arm

Stěpánek and coworkers characterized PEO-PCL micelles with larger PCL blocks (5, 13, and 32 kDa) at a constant PEO block length of 5 kDa with static (SLS) and dynamic light scattering (DLS).[23] Scattering intensities and relaxation time distributions from DLS and SLS data do not indicate any dissociation dependence on concentration at low concentrations (i.e. kinetically frozen). The longer PCL blocks stabilize the micelles as is often evident through lower CMC values (Table 1). The

geometry, block arrangement and functional groups substituted onto PCL also influence the CMC of micelles containing PEO and PCL blocks.

As expected, hydrodynamic radii and radii of gyration were comparable and thus indicative of a spherical shape. Average-density aggregation numbers (Z) were calculated from hydrodynamic radii to range from 2,300-10,900 for PCL blocks 5-32 kDa in length. These aggregation numbers were consistent with other micelles known to form vesicles at similar sizes. This implies that the inner core was liposome-like, or hollow. The diameter of these micelles increased from 80-200 nm across PCL blocks of 5-32 kDa as expected. Size distributions are normally characterized on many micelles.

### 1.3.1 CMC measurements

Another common characterization of these micelles is the CMC, or the lowest concentration of copolymer required to form a micelle. This quantity indirectly describes the equilibrium and stability and is measured with fluorescent excitation intensity ratios of pyrene at 338-339 nm to 333-334 nm as a function of polymer concentration. As the excitation intensity increases, pyrene enters an environment of lesser polarity. Thus pyrene is being encapsulated inside the lipophilic core of the micelle. The CMC occurs at the onset of the increase in intensity as a function of polymer concentration.

### 1.3.2 Relative core microviscosity

As mentioned previously in Stěpánek's characterization, when the polymer concentration dependence of the longer PCL block micelles is absent at low concentration, the micelle is considered to be kinetically frozen. In this case, the equilibrium will ultimately shift to form unimers. However the core is so entangled and viscous with the larger PCL blocks, the time scale of the light scattering experiments could not capture this. The relative core microviscosity of micelles can be estimated based the fluorescent emission intensity ratio of 1,3-bis-(1-pyrenyl)propane (dipyrene) and pyrene at 480 nm and 390 nm, respectively. Dipyrene forms an excimer when rotation is free around an alkyl chain between two pyrenes to change conformation and emits light at 480 nm ($I_E$). The emission of light from pyrene at 390 nm ($I_M$) (in competition with dipyrenes emission at 480 nm) is used as a reference to the excimer.[38] As the core becomes viscous, change is conformation is restricted, and the emission intensity at 480 nm decreases, and the ratio $I_M/I_E$ increases. It should be noted that the $I_M/I_E$ or $I_E/I_M$ ratios are used interchangeably. These types of characterizations are useful in understanding release profiles of a model drug amongst a series of copolymers as well as the thermodynamic stability of micelles.

## 2.0 Drug Delivery from Diblock PEO-PCL Copolymer Micelles

In addition to knowledge of the thermodynamic stability of these micelles, understanding the thermodynamic affinity of a drug for the PCL core is also crucial for drug delivery. The impact of core length on release, biocompatibility, and efficacy *in vitro* in cell culture has been studied with a variety of lipophilic drugs, especially anti-cancer agents. Indomethacin, a hydrophobic non-steroidal anti-inflammatory drug (NSAID) was the first model drug encapsulated in diblock PEO-PCL micelles.[39] As expected, CMC's decreased with increasing hydrophobic chain length and were on the order of $10^{-7}$ M (Table 1.). A 40% encapsulation efficiency was achieved with indomethacin in PEO-PCL micelles with block molecular weights of 5 kDa and ~5.5 kDa. Release studies showed indomethacin to release in a Fickian profile over 12-14 days without a burst effect.[40] This work also showed that the micelle size increased when higher concentrations of drug were loaded, as has been seen with other drugs (fenofibrate and amiodarone) in PEO-PCL micelles.[22, 41] As shown in Table 2, the micelle size generally increases when the hydrophobic solute (drug) is retained in the core. This is expected as the PCL chains in the core will have a relatively small free volume (due to their crystallinity), and the inclusion of drug molecules will increase the core size, and thus the micelle size.

Allen *et al.* were first to study the biocompatibility of $PEO_{44}$-$PCL_{20}$ micelles (Subscripted numerals on $PEO_{44}$-$PCL_{20}$ represent 44 ethylene glycol, and 20 ε-caprolactone units) with PC 12 (rat neuroendocrine tumor) and MCF-7 breast cancer

cells.[42] $PEO_{44}$-$PCL_{20}$ micelles with a PCL block length of 2.3 kDa, showed little cell death over a range of concentrations in the PC 12 and MCF-7 cell lines. Yet a $PEO_{44}$-$PCL_{14}$ micelle with a PCL block length of 1.7 kDa induced 10-20% cell death over the same range of concentrations. This implies that the smaller micelle ($PEO_{44}$-$PCL_{14}$) may have internalized in the cell and disrupted the cells essential metabolic

Table 2. Influence of encapsulated drug on PEO-PCL micelle size

| PEO MW (kDa) | PCL MW (kDa) | Drug | Encapsulation efficiency (%) | Micelle size without drug (nm) | Micelle size with drug (nm) | Ref. |
|---|---|---|---|---|---|---|
| 5.0 | 6.2 | indomethacin | 40 | 130 | 165 | [40] |
| 5.0 | 1.0 | fenofibrate | 29 | 19 | 22 | [22] |
| 5.0 | 2.5 | fenofibrate | 95 | 22 | 24 | [22] |
| 5.0 | 4.0 | fenofibrate | 92 | 33 | 41 | [22] |
| 5.0 | 5.0 | CsA | 52 | 88 | 100 | [45] |
| 5.0 | 13 | CsA | 64 | 79 | 99 | [45] |
| 5.0 | 24 | CsA | 50 | 100 | 102 | [45] |
| 2.0 | 2.0 | DOX | n/a | 17 | 25 | [25] |
| 5.0 | 2.5 | DOX | n/a | 30 | 23 | [25] |
| 5.0 | 5.0 | DOX | n/a | 41 | 37 | [25] |
| 5.0 | 8.5 | DOX | n/a | 57 | 84 | [25] |
| 5.0 | 25 | DOX | n/a | 86 | 105 | [25] |
| 5.0 | 6.0 | rapamycin | 24 | 31 | 48 | [60] |
| 5.0 | 6.0 | rapamycin + 1:20 α-tocopherol | 50 | 63 | 44 | [60] |
| 5.0 | 10 | rapamycin | 50 | 79 | 76 | [60] |
| 5.0 | 10 | rapamycin + 1:20 α-tocopherol | 73 | 109 | 107 | [60] |
| 5.0 | 18 | rapamycin | 31 | 40 | 46 | [60] |
| 5.0 | 18 | rapamycin + 1:20 α-tocopherol | 66 | 57 | 44 | [60] |

functions. This will be explored further in a following section. In the same study, *in vitro* release of FK506 (an immunosuppressive and neural outgrowth factor), and its synthetic analogue L-685,818 from the $PEO_{44}$-$PCL_{20}$ micelles did not demonstrate enhanced differentiation in PC 12 cells without NGF (nerve growth factor) as was expected. Later work *in vivo*, demonstrated $PEO_{44}$-$PCL_{20}$ micelles containing FK506 promoted a faster recovery in Hanover-Wistar rats with crushed peripheral nerve lesions than simply injecting FK206 at the same concentration.[43] In comparison to the indomethacin loaded PEO-PCL micelles, the CMC of $PEO_{44}$-$PCL_{20}$ was decreased to $10^{-8}$ M, and thus its *in vivo* stability was increased by increasing the length of PCL block relative to the PEO block. Dihydroxytestosterone (DHT), another highly lipophilic molecule was also encapsulated with the $PEO_{44}$-$PCL_{20}$ micelle to study its release and biological activity upon release.[44] Similar to the release of indomethacin, DHT released in a Fickian manner and at a slower rate when the loaded concentration was increased. To assess the biological activity of DHT upon release, HeLa cells were co-transfected with MMTV-Luciferase and androgen receptor (AR). If DHT is still biologically active when it releases from the $PEO_{44}$-$PCL_{20}$ micelles, then it will bind to AR and induce transcription of the luciferase gene and ultimately the translation of luciferase. This work demonstrated that the biological activity of free DHT and micelles containing DHT were equivalent in terms of luminescence per μg cellular protein based on a luciferase assay. Therefore, the biological activity was retained upon release in cell culture.

The hydrophobic model drugs discussed until now utilized dialysis to remove free drug. This typically works for most model drugs and PEO-PCL micelles, however in the case of fenofibrate approximately 75% of the initially loaded model released during dialysis.[22] This can be overcome by loading polymer and fenofibrate in ACN, and approaching the azeotrope of water –ACN through the addition of water. The ACN can then be removed through solvent evaporation under vacuum. High loading efficiencies as great as 95% were achieved, and this efficiency was shown to have an optimal PCL block length. With constant PEO block length (5 kDa), the PCL block at 2.5 kDa loaded 95% fenofibrate, 4 kDa 90%, and 1 kDa only 30%. It would have been expected that the loading capacity would increase as the PCL block length increases. Thus the PCL block length should be optimized to obtain the highest encapsulation efficiency for each prospective drug.

Another instance of solvent evaporation in drug loading of PEO-PCL is cyclosporine A (CsA), an immunosuppressive agent with low water solubility.[45] Three different polymers were compared amongst loading capacity, release, and size, each with a constant PEO block molecular weight of 5 kDa, and PCL block molecular weights of 5, 13, and 24 kDa. Utilizing different solvents during solvent extraction, Aliabadi *et al.* were able to show that THF gave smaller micelles (40-86 nm in diameter) compared to acetone which gave 80-100 nm diameter micelles amongst the same series of PEO-PCL polymers. The release profile of CsA in PEO-PCL(13 kDa) was evaluated in comparison to the current formulation of CsA in Cremophor® EL (Sandimmune®) in phosphate-buffered saline (PBS) with 4% bovine serum albumin

(BSA). The Cremophor® EL formulation depicted a burst type release with approximately 80% of the drug cumulatively released at 12 h. While the PEO-PCL formulation showed a more sustained release: releasing only 10% over the same time frame. The PEO-PCL block co-polymer had a lower CMC of $6 \times 10^{-7}$ M compared to Cremophor® EL ($4 \times 10^{-5}$ M), and a lower fluorescent intensity ratio ($I_E/I_M$) of the excimer to monomer of dipyrene to pyrene (0.11-0.19). This demonstrates that the relative micelle core viscosity was higher in PEO-PCL, and thus diffusion of CsA was limited and the release was sustained.

The same series of PEO-PCL polymers were investigated with respect to their ability to encapsulate and release an amphiphilic drug, Amiodarone (AMI) is an anti-anginal and anti-arrhythmic drug used in the therapy of ischemic heart disease.[41] Both, a commercial formulation of the surfactant polysorbate 80 with benzyl alcohol, and free AMI precipitate upon immediate dilution, and induce red blood cell hemolysis. Increasing the length of the PCL block increased the loading amount of the amphiphilic drug AMI and reduced hemolysis in Sprague-Dawley rats. Only 20-40% of AMI (2-5 mg loaded) precipitated after release from PEO-PCL(13 kDa) micelles, upon a 2X dilution into PBS at 37°C compared to 100% precipitation for free AMI. However, it should be noted that when 5 mg of AMI was loaded in PEO-PCL micelles, the amount precipitated nearly doubled that of 2 mg AMI being loaded. Hemolysis from the PEO-PCL(13 kDa) micelle loaded to 2 mg AMI was reduced to 1/10 the damage caused by free AMI over a two order magnitude concentration range. When this same micelle was loaded to 5 mg AMI, the extent of

hemolysis was equivalent to free AMI. Thus, both the amount of drug loaded should and PCL block length should be optimized in preformulation of prospective drug candidates. So far it is evident that PEO-PCL diblock co-polymers are:

- biocompatible with multiple cell lines
- provide extended release over currently approved formulations
- can retain a drugs biological activity *in vitro* and *in vivo*
- can prevent aggregation and protect red blood cells from hemolysis

## 2.1 Drug Delivery of Anti-Cancer Agents from Diblock PEO-PCL Micelles

Since PEO-PCL micelles can protect from some unwanted drug side effects, it is useful to understand how they deliver cytotoxic, hydrophobic drugs often used in chemotherapy. One of the most widely used chemotherapeutic agents is doxorubicin (DOX), an anthracyline antibiotic which induces apoptosis in tumor-specific cells.[46-49] A series of PEO-PCL polymers were synthesized with varying PCL block lengths (2-24.7 kDa) to investigate the cytotoxicity of these micelles and their ability to load and release DOX.[25] The loading content of these micelles ranged only 3-4 wt% of DOX amongst this series of PEO-PCL micelles. The PCL core over this range of molecular weight did not increase the loading capacity as seen with other model drugs. However, no detectable hemolysis was induced at concentrations as high as 200 μg/mL DOX in PEO-PCL(5 and 24.7 kDa), compared to 11% hemolysis at the same concentration for free DOX. Dynamic light scattering showed

substantial increases in polymer size with increases in PCL block length (22-100 nm in diameter). At pH 7.4, DOX was cumulatively released slightly faster (~15%) with a 24.7 kDa PCL block compared to a 5 kDa (~10%) over 35 days. At pH 5, the cumulative release was accelerated to 80% in the 24.7 kDa block, and to 60% in the 5 kDa block over the same time frame as pH 7.4. Slightly acidic conditions may have increased the rate of ester degradation in the core. However, it is more likely that the increase in release rate is due to the octanol-water partition coefficient (lipophilicity) reducing dramatically from 5.1 to 0.2 (pH 7.4 to pH 5).[50] The fact that the larger block length accelerated release at both pHs, suggests that the PCL core viscosity was reduced, potentially due to an increased volume of core. This unexpected release behavior will be analyzed further with respect to thermodynamic affinity. It is important to notice the increased rate of release at lower pH in relation to cancer therapy because tumor microenvironments are often slightly hypoxic.

Paclitaxel is another natural product that is considered to be the most successful in the treatment of breast, ovarian, and lung cancers, by both arresting mitotic function and phosphorylating and thus inactivating the anti-apoptotic Bcl-2 and Bcl-xL proteins.[51, 52] Paclitaxel loading, cell culture release, and biocompatibility studies have been investigated with a series of PEO-poly(δ-valerolactone) (PVL) micelles by Allen and coworkers.[53] δ-Valerolactone is identical to ε-caprolactone except the lactone ring contains only 5 carbons instead of 6 in ε-caprolactone, thus it is slightly less lipophilic. This slight reduction in

lipophilicity may account for the higher CMC's $10^{-5}$-$10^{-6}$ M over a range of PVL block lengths (0.5-5 kDa) at two different PEO block lengths (2 and 5 kDa). The reason the reduction in lipophilicity may increase the CMC is because the micellization is based on the net difference in thermodynamic affinity between the water and PEO, and the difference of PVL and other PVL chains. The greater the affinity of the core chains for themselves induces a lower CMC. The PEO-PVL copolymer with both blocks being 2 kDa in molecular weight was found to retain the highest paclitaxel loaded concentration (9 mg/mL) following centrifugation and sterile filtration. This result was expected because all loaded micelles except this polymer had diameters on the order of magnitude of the filter. Thus filtration could have physically disrupted the micelle structure. These co-polymers were shown to be biocompatible with a CHO-K1 cell line up to concentrations of 1 g/L. This micelle formulation increased paclitaxel aqueous solubility 9000-fold, and was found to biologically active upon release in MCF-7 breast and A2780 ovarian cancer cell lines. Though the loading potential of this class of micelles would only require 28 mL of a 10wt% copolymer solution for ovarian cancer and Kaposi's sarcoma treatment, lesser administered volumes would be desired.

In order to potentially increase paclitaxel loading and decrease this administered volume, PEO-PCL polymers have been synthesized with a terminal maleic cap on the PCL end to crosslink the core.[54] This maleic-capped diblock was then activated with thionyl chloride, and subjected to radical polymerization with a

potassium peroxodisulfate initiator. A series of diblock and triblock copolymers were synthesized with PEO block lengths of 2 and 5 kDa, and PCL blocks lengths ranging from 1.2-18 kDa. As the PCL block length increased, the paclitaxel loading efficiency increased as expected. As much as 85% of the loaded paclitaxel was encapsulated in a diblock PEO(5kDa)-PCL(18kDa) co-polymer. Though triblock copolymers will be covered in a following section, it is worthwhile to note that 72% of paclitaxel was loaded into the PEO(5kDa)-PCL(18kDa)-PEO(5kDa). It is likely that a PCL(18kDa)-PEO(5kDa)-PCL(18kDa) triblock would be superior in paclitaxel loading. However, this copolymer upon radical polymerization would likely phase separate from solution. Therefore, the diblock copolymer with the largest PCL core would remain the best option for a potential paclitaxel formulation. Aside from paclitaxel, there are some anticancer compounds that have a series of derivatives which each have different levels of cytotoxicity.

Inhibitors of STAT3 oncogenic signal transduction, or cucurbitacins have an interesting trend where their derivatives cytotoxicity is linearly related to its hydrophobicity.[55] In this case, it may be feasible to utilize the same core with different block lengths for a series of derivatives. Molavi *et al.* investigated the release, and *in vitro* and *in vivo* activity of cucurbitacin I and B from PEO-PCL and poly(α-benzyl carboxylate ε-caprolactone)(PBCL)-PEO micelles. The addition of a benzylic carboxylate functional group to the PCL core increases the lipophilicity, and thus smaller block lengths compared to conventional PCL may facilitate similar

thermodynamic affinities of the target drug to the core. A series of diblock copolymers employing a PEO block length of 5 kDa, PCL blocks of 5 or 24 kDa, or PBCL at 4.7 kDa were utilized to encapsulate cucurbitacin I and B. Cucurbitacin B differs only in an acetyl group in place of a hydroxyl at the 25' position of cucurbitacin I.[56] Therefore cucurbitacin B has an axis of symmetric polarity, and is slightly more lipophilic than cucurbitacin I. The loading efficiency increases with PCL block length for both derivatives (~30-65%). However a substantial increase in efficiency occurs with cucurbitacin B in the 4.7 kDa PBCL core (92%) over cucurbitacin I (74%). This increase could be owed to the fact that cucurbitacin B is more lipophilic. All release profiles were Fickian, and the PEO-PCL (5 kDa) and PEO-PBCL (4.7 kDa) profiles were nearly identical for both derivatives. At 8 hours, approximately all of the cucurbitacin I had been released, whereas cucurbitacin B only cumulatively released 40-60%. This sustained release can also be owed to the reduced polarity of cucurbitacin B. In each derivative the 24 kDa PCL core exhibited a slower release compared to the 5 kDa PCL and PBCL cores, and this is may be due to their increased thermodynamic affinity for the core. Cucurbitacin I released from PEO-PCL(24 kDa) was more potent with an $IC_{50}$ of 10 μM versus $IC_{50}$ of 50 μM for cucurbitacin B against a B16.F10 cell line (melanoma of C57/black origin) after 24 h. In each derivative the $IC_{50}$ of free cucurbitacin in methanol solutions was approximately half of the $IC_{50}$ of cucurbitacin released from the PEO-PCL micelles. However, there was little statistical difference in terms of *in vivo* tumor volume upon

treatment with free and PEO-PCL encapsulated cucurbitacin I as both virtually abolished growth. If a targeting moiety to the tumor (e.g. EGF [57], Her2/neu [58], PSMA [59]) was desired to cover the micelle and enhance *in vivo* activity, the micelle size may need to be reduced. Thus, the core block length would need to be shortened. However, this may result in faster release, as was seen with the cucurbitacins. Therefore additives can be used to improve encapsulation efficiency and potentially the release of the drug from the core while maintaining *in vivo* activity.

Rapamycin, an inhibitor of a key regulator in cell proliferation in solid tumors has been efficiently encapsulated into PEO-PCL micelle cores with α-tocopherol.[60] In this study a series of PEO-PCL micelles with a constant PEO block length of 5 kDa, and varying PCL block lengths of 6,10, and 18 kDa were used to encapsulate and release rapamycin. α-tocopherol, or vitamin E is more lipophilic than a single unit of caprolactone, and will partition into the PCL core and provide more thermodynamic affinity for rapamycin to the core. This was necessary because rapamycin is extremely lipophilic with an octanol water partition coefficient of 5.8. As expected, the CMC of these micelles decreased with increasing PCL block length and were on the order of $10^{-7}$-$10^{-8}$ M. The addition of α-tocopherol in molar ratio of 1:10 and 1:20 to PEO-PCL demonstrated negligible increases in the CMC. However, it should be noted that addition of α-tocopherol beyond the ratio of 1:20 shifted the phase equilibria of the PEO-PCL-water system to precipitate as particles greater than 400 nm. This precipitation phenomenon would not necessarily be recognized in the

fluorescence emission spectra of pyrene. Encapsulation efficiencies were increased with 1:20 molar ratios of α-tocopherol in each PEO-PCL micelle, with an apparent optimum PCL block length of 10 kDa resulting in 72% efficiency. The PEO-PCL micelles with 18 kDa PCL blocks encapsulated only 66%. As the reduction in efficiency compared to the 10 kDa block was unexpected, it foreshadowed precipitation in release studies. Thus the addition of rapamycin to PEO-PCL micelles loaded with α-tocopherol further shifted the phase equilibria of the PEO-PCL-water system to precipitate as seen with higher molar ratios (>1:20 α-tocopherol). The release of rapamycin was not significantly impacted by α-tocopherol. However, the use of 4% BSA in the release medium did taper off the release, and this was likely due to an osmotic pressure effect enhancing the interaction of the rapamycin with the core. In some cases, the addition of hydrophobic molecules may not provide and extended release or increased encapsulation. Also, the most efficient design of a core for natural product delivery is not always intuitive, as was the case of DOX in which a larger core actually accelerated the release. This trend was contrary to the release of other anticancer compounds with increased PCL block lengths. Since all of these compounds are usually polycyclic lipophilic compounds with a slight dipole, it is useful to resolve this disparity, and gain an understanding of drug-core affinity for future core designs.

## 2.2 Flory-Huggins Interaction Parameters between drugs and polymeric cores

Flory-Huggins interaction parameters have been employed to evaluate the thermodynamic affinity of geldanamycin prodrugs, amongst other anti-cancer agents for PCL cores.[33] Geldanamycin (GA) (Figure 3) is known to inhibit the ATP binding pocket of Hsp90, which reduces a cancer cells ability to refold essential proteins and eventually survive. The interaction parameter analysis facilitated the design of a novel type of prodrug, and illustrated how increasing lipophilicity of a target drug can increase the micelle loading capacity, and ultimately the aqueous solubility. Using functional group contributions of the drug and PCL chain, partial solubility parameters were estimated, and Flory-Huggins interaction parameter was calculated. When this interaction parameter approaches zero, the PCL chain is completely solvated with drug. A series of GA derivatives were synthesized with varying lengths of aliphatic chains at the 17' position, and were evaluated with MCF-7 breast cancer cells to gauge the potency relative to GA. Most of these derivatives cytotoxicity was reduced at least an order of magnitude, however one bromoester derivative, 17-(ethylamino-2-bromododeconate)-17-demethoxygeldanamycin (GA 17'C16Br) (Figure 3.) $IC_{50}$ reduced by only a factor of 5 (110 nM). As shown in Figure 3 b, the ethylamine ester at the 17' position will hydrolyze *in vivo* to activate the drug, and the addition of the electron withdrawing group (Br) increases the lability of the ester bond, decreasing the half life.

GA was poorly soluble in PEO(5 kDa)-PCL(10 kDa) micelles, however GA 17'C16Br's solubility was increased 2 orders of magnitude over GA and its loading efficiency approached 100% in the same micelle. Release studies of GA 17'C16Br at physiological conditions demonstrated a Fickian sustained release over 2 weeks. The Flory-Huggins interaction parameter is a useful tool to understand thermodynamic affinity of a drug for a micelle core. As the interaction parameter would likely decrease with increased molecular weight of the PCL block[61], it is likely that DOX has a higher value interaction parameter than cucurbitacin with PCL.

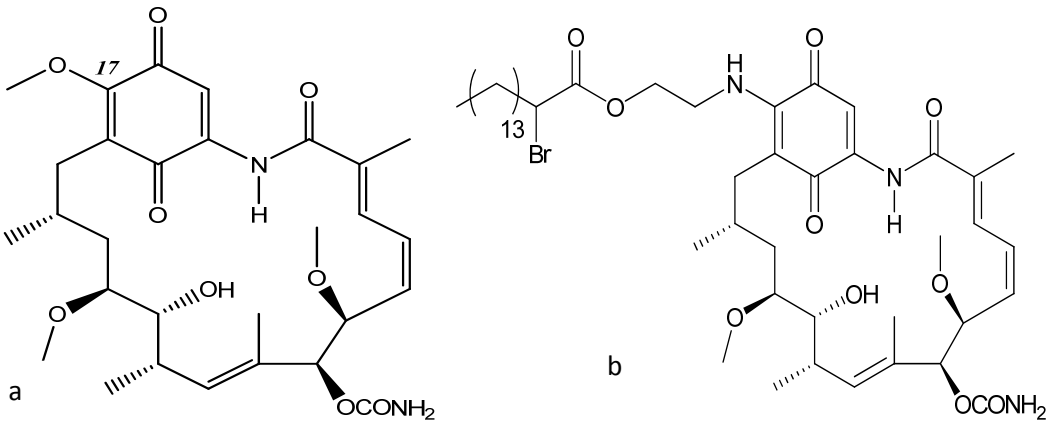


Figure 3. (a) Geldanamycin, and its (b) bromoester prodrug, GA 17'C16Br.

From these studies on the release of anticancer agents, much can be inferred from the release characteristics resulting from the thermodynamics of interaction for the design of future PCL cores. However only so much can be done to alter the thermodynamics of interaction between a target drug and a diblock PEO-PCL micelle. Therefore triblock copolymer micelles of PCL-PEO-PCL, star copolymers, as well as micelles with substituted PCL blocks have been developed.

## 2.3 Drug Delivery from Triblock Copolymer Micelles of PEO and PCL blocks

The first triblock PCL-PEO-PCL micelles used as a delivery vehicle were used to characterize the drug loading capacity and release of the model drug clonazepam.[62] Three triblock copolymers were investigated with each PCL chain being 2.4, 3.8, or 8.5 kDa and the PEO block of 8 kDa was constant amongst this series. The synthesis and micelle preparation of the triblock copolymers was similar to that of diblock PEO-PCL micelles, except the initiation of polymerization occurred on each end of the PEO chain. The loading efficiency of clonazepam was shown to range from 12-26% depending on the initial organic solvent. As expected the loading efficiency increased with PCL block length. Also similar to the diblock PEO-PCL micelles, these triblock micelles had CMC's on the order of $10^{-7}$-$10^{-8}$ M and were 20-30 nm in diameter. Clonazepam was cumulatively released in a sustained Fickian manner over a period of 4 days. Similar release behavior has been seen in other triblock micelles of PEO, PCL, and PVL blocks with nimodipine,[63] indomethacin,[64] and 4'-demethylepipodophyllotoxin (DMEP).[65] Though the triblock micelles have larger lipophilic cores, the drug loading efficiencies, capacities, and release behavior are not substantially improved compared to diblock PEO-PCL micelles. Therefore micelles with more dense lipophilic cores have been developed, such as substituted PCL blocks and star copolymers.

## 2.4 Drug Delivery from Star Copolymer Micelles of PEO and PCL

Three and Four arm star copolymers with PCL as the center of the star and PEO conjugated to the ends have were characterized in terms of their size distribution and CMC.[66] Polymerization for the PCL block was initiated off of pentaerythritol terminating with a hydroxyl, for the 4 star copolymers (Figure 4). A carboxylic acid was then added to one end of the PEO chain through reacting succinic anhydride with a terminal hydroxyl. The PEO's carboxylic acid was then attacked by the hydroxyl of the 4 star PCL block. For 3-star armed copolymers, diblock PEO-PCL copolymers were conjugated to the 1,3,5-Benzenetricarboxylic acid (initiator) through PCL, after activating the initiator with thionyl chloride. The diblock PEO-PCL copolymers used for the 3 star-armed copolymers had CMCs on the order of $10^{-7}$ M, and the CMC of the 3 and 4 star copolymers was sequentially lowered from 5 x $10^{-8}$ M to 2 x $10^{-8}$ M, respectively. This study demonstrated that effectively increasing the number of arms branched off of the core did increase the stability of the micelles through lower CMCs. It should be noted that when the ends of the star copolymers have PCL blocks with large enough molecular weights and the center is PEO; these polymers can gel and even phase separate in aqueous solution.[67]

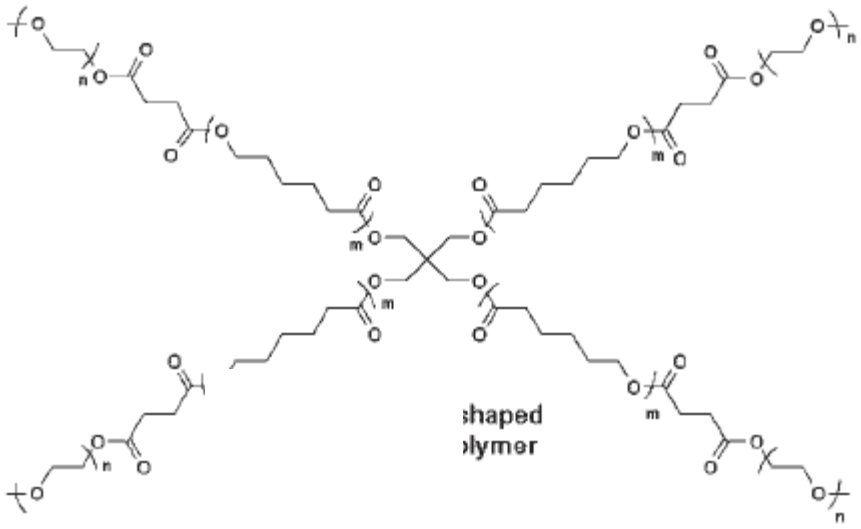

Figure 4. Structure of 4-arm star PEO-PCL block copolymer. From reference [66].

Another study evaluated the drug delivery potential of 4 arm PCL-PEO star copolymers with all-trans-retinoic acid (*at*RA).[34] This work characterized a 4 arm center block of PCL (19.6 kDa) with 2 kDa PEO conjugated to each end. Micelles were formed by drop wise precipitation from organic solvent into water, or dry polymer in water being sonicated. Sonication produced larger micelles (144 nm in diameter compared to 77 nm) and had higher loading efficiencies (10% compared to 6%). Additionally, micelles produced by sonication extended the cumulative release of *at*RA to 40% at 170 h, compared to the complete release of *at*RA in drop wise prepared micelles over the same time frame. To further increase the stability of these micelles, Allen and colleagues developed 6 arm-star copolymers, using PVL in the center of the star with PEO at the terminal ends.[32] Polymerization of PVL was initiated off of dipentaerythritol and PEO was coupled to the PVL ends. These

micelles had somewhat lower CMC's on the order of $10^{-6}$ M than the diblock PEO-PVL micelles. As much as star copolymers have contributed to our knowledge of the range of copolymer block geometries that will form micelles, the development to this point has only slightly increased their stability. Therefore, PEO-PCL diblock copolymers with functional groups coupled to the PCL were developed.

## 2.5 Drug Delivery from Substituted Diblock PEO-PCL Copolymer Micelles

Lavasanifar and coworkers synthesized α-substituted ε-caprolactone monomers (Figure 5) with functional substituents of benzyl carboxylate or carboxylic acid to produce PEO-poly(α-benzyl carboxylate ε-caprolactone) (PBCL) and PEO-poly(α-carboxylate ε-caprolactone) (PCCL).[68] It should also be noted that PCL blocks can also have functional groups such as hydroxyls, carboxylic acids and epoxides grafted onto the chain post-post polymerization via atom transfer radical addition reactions or Michael-type additions.[69-71] The addition of benzyl carboxylate or carboxylic acid demonstrated how the CMC can be tuned within a PEO-PCL copolymer with nominally the same block lengths. A 5 kDa PEO block was used for each copolymer prepared. With a PBCL block length of 2 kDa an 8 x $10^{-8}$ M CMC was achieved. However a typical PEO-PCL diblock with a PCL block length of 5 kDa had a CMC double that of the PEO-PBCL diblock with more than twice the length. This implies that the addition of a benzyl carboxylate function group to a PCL core can reduce the CMC by roughly a factor of ¼. Conversely, the

addition of a carboxylic acid functional group to a slightly smaller PCL block (1.8 kDa) increased the CMC of the PEO-PCL diblock by 2 orders of magnitude. It should be noted that the addition of the benzyl carboxylate function group increased the micelle size to 60 nm over the typical PEO-PCL(5 kDa) diblock (40 nm). The ability to tune the CMC by adjusting the functional groups on the core implies that the thermodynamic affinity of a drug for the core should change as well.

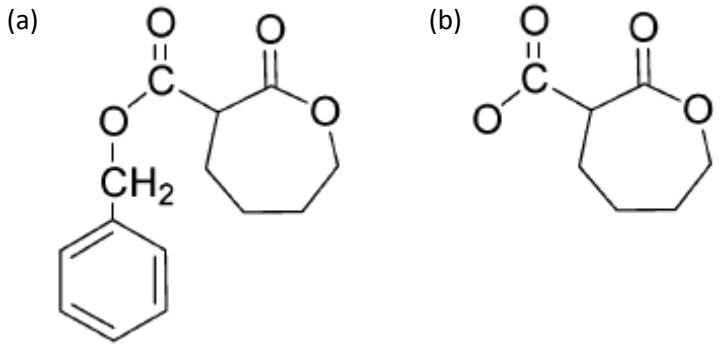


Figure 5. (a) α-benzyl carboxylate ε-caprolactone and (b) α-carboxylate ε-caprolactone monomers. From reference [68]

Compared to PEO-PCL (24 kDa PCL block), a PEO-PBCL micelle with 1/5 of the PCL block length increased the encapsulation efficiency of cucurbitacin B almost 30%. However, the cumulative release from the PBCL core was slightly faster than the 24 kDa PCL core. Nonetheless, the increases in stability and encapsulation efficiency for some drugs are superior to typical diblock PEO-PCL copolymers. In addition to characterizing the drug delivery properties of PEO-PCL micelles, the fate of micelles during and following release *in vivo* is also significant.

## 3.0 Characterization of Cellular Uptake and Pre-Clinical Efficacy *in vivo*

As a first step in understanding what happens to these micelles *in vivo*, the micelle-cell interaction was studied extensively *in vitro* in multiple cell lines. It is well known that when PEO chains reside at the corona, they impart negative zeta potentials on the micelles.[72-74] A smaller PEO chain would increase the magnitude of this negative zeta potential because the layer of bound water is smaller than that of micelles with larger PEO chains. Negative zeta potential increases the micelles ability to be taken up by macrophages of the mononuclear phagocyte system.[74, 75]

### 3.1 Cellular Internalization of PEO-PCL micelles

As this macrophage uptake would represent one mode of the micelles clearance *in vivo*, it is also important to understand how these micelles interact with other cells. This question has typically been addressed through the use of fluorescent dyes or radioactive compounds. Both of these approaches were used to demonstrate that the cellular uptake of $PEO_{44}$-$PCL_{20}$ micelles in a PC 12 cell line was temperature and pH dependent.[76] These temperature and pH dependences on the uptake are indicative of endocytosis mechanisms. Mahmud *et al.* studied the uptake of various PEO-PCL micelles with different PCL block lengths in MCF-7 breast cancer cells.[21] These micelles were loaded with a lipophilic fluorescent dye, DiI, and the mechanism of endocytic uptake was investigated with chlorpromazine and cytochalasin B. An optimal PCL block length of 13 kDa, and PEO block length of 5

kDa demonstrated the highest cellular uptake. Chlorpromazine is known to inhibit clathrin-mediated endocytosis by reducing clathrin-coated pit formation at the cellular membrane, while cytochalasin B inhibits phagocytosis through disruption of microfilament bundles at the cell membrane. Both endocytosis inhibiting agents reduced uptake at the optimal block lengths of PEO and PCL. Incubation of MCF-7 cells at 4°C in the absence of these agents also reduced uptake. This suggests an energy-dependent uptake mechanism, which further supports both clathrin-mediated endocytosis and phagocytosis.

## 3.2 Cellular Distribution of PEO-PCL micelles

Further work investigated the cellular distribution of $PEO_{45}$-$PCL_{23}$ micelles with tetramethylrhodamine-5-carbonyl azide (TMRCA) conjugated to the end of the PCL block in PC 12 and NIH 3T3 cell lines.[77] Though PEO is known prevent protein adsorption, and had been shown to reduce uptake of PEO:PLGA nanoparticles in human monocytes,[78] not all of the PEO-PCL micelles were excluded from these cell lines. Using a series of organelle and nuclear selective dyes, it was found that PEO-PCL was primarily taken up into cytoplasmic organelles, specifically the Golgi apparatus and mitochondria. Shuai *et al.* also saw DOX with PEO-PCL micelles localized to the cytoplasm, as opposed to free DOX localized in the nucleus.[25] Cisplatin, another anti-cancer agent was encapsulated with 73% efficiency into PEO-PCL micelles and was taken up into SKOV-3 ovarian cancer cells. [72] However its

localization to endocytic vesicles in the cytoplasm retarded its potential cytotoxicity compared to pH sensitive micelles localizing the drug in the nucleus. Thus PEO-PCL micelles are endocytosed into a variety of cell lines, and typically localize in endocytic vesicles in the cytoplasm. The localization of these micelles and there cargo into the cytoplasm is beneficial to know prior to *in vivo* studies. Though the majority of the drug is likely be released independent of cellular internalization, if the drug requires localization in the nucleus, there may be some reduced efficacy *in vivo*.

## 3.3 Efficacy and Stability of PEO-PCL micelle drug delivery *in vivo*

Thus far, delivery of various drugs have shown sustained release from PEO-PCL micelles over free drugs, and have shown efficacy *in vitro* in cell culture. However it is common for drugs to show efficacy in the lab, but display cytotoxicity and reduced efficacy in animals. Therefore it is important to gauge the use of these micelles *in vivo*. Lavasanifar and coworkers examined the pharmacokinetics and biodistribution of CsA released from PEO-PCL micelles in Sprague-Dawley rats compared to the current formulation in Cremophor® EL.[79] The PEO-PCL micelles with PCL block lengths of 5 and 13 kDa both encapsulated approximately 30% of the drug and were nominally 100 nm in saline. These micelles increased both the blood and plasma CsA concentrations approximately 6 fold and reduced the volume of distribution and clearance 10 and 8 fold, respectively. The pharmacokinetics of these micelles imposed lower concentration distributions of CsA in all organs except the heart. Most paramount, the PEO-PCL micelles reduced the concentration in the

kidneys. This is important because CsA exerts it toxicity in that organ. Rapamycins pharmacokinetics and tissue distribution in rats was investigated upon release from PEO-PCL micelles with and without α-tocopherol, compared to the current solution formulation of Tween 80/PEO400/N,N dimethylacetamide.[80] The blood and plasma concentrations were not significantly increased with the PEO-PCL micelles with or without α-tocopherol. However, the micelles with α-tocopherol did slightly reduce the volume of distribution and clearance. Additionally, the organ distribution of rapamycin was significantly reduced in PEO-PCL micelles with α-tocopherol liver, kidney, spleen, brain, lung, and heart. PEO-PCL micelles optimally reduced rapamycin distribution compared to micelles with α-tocopherol and the control formulation. As rapamycin demonstrates acute neurotoxicity, it is worthwhile to note its significant decrease in the brain with PEO-PCL micelles. Also important to the administration of micelle-delivered drugs is the fate of the micelle.

### 3.3.1 Fate of PEO-PCL micelles *in vivo*

To prevent a burst release *in vivo*, it is important that the micelles are stable. The equilibrium between unimers and micelles *in vivo* as well as the CMC are necessary to understand the proper polymer concentration administered. Liu *et al.* have evaluated this equilibrium for PEO-PCL micelles in Balb/C mice.[17] Three PEO-PCL micelles with a constant PCL block length of 5 kDa and PEO block lengths of 2, 5, 10 kDa were evaluated. The PEO-PCL with a PEO block length of 5 kDa was the most stable retaining a monomodal size distribution according to DLS at

physiological conditions with 4.5% BSA over 10 days. PEO-PCL copolymers were incubated with mouse plasma for 5 h and 37°C at concentrations an order of magnitude below the CMC. No significant interactions between the plasma and micelles were found. Tritium labeled [$^{3}$H] PEO-PCL micelles were injected into the tail vein of Balb/C mice and samples were analyzed via a scintillation counter. At copolymer concentrations (250 mg/kg) 70 fold above the CMC (upon dilution in the mice), 74% of the micelles were intact in the plasma after 24 h according to gel filtration chromatography. When the copolymer concentration (2 mg/kg) was 2 fold below the CMC upon dilution, 55% of the micelles remained intact after 24 h. The tissue distribution of PEO-PCL at 250 mg/kg and 2 mg/kg remained at approximately 10% of the initial dose for each concentration in the liver through 48 h. Other tissue distribution studies of PEO-PCL micelles have noted the increased uptake in the liver due to their size being less than 70 nm.[81] Between 1% and 5% of the initial dose was retained in the heart, kidney, spleen, and lung through 48 h for the same doses. However when 0.2 mg/kg PEO-PCL was administered, the accumulation in the liver rose from 10% of the initial dose to approximately 40% through 48 h. Between 5% and 10% of the initial dose remained in the heart, kidney, spleen, and lung through 48 h. The higher retention of this dose (0.2 mg/kg) in organs may be a reflection of the pharmacokinetics. At 0.2 mg/kg, the PEO-PCL micelle was rapidly cleared from the plasma in approximately 12 h. However, the 2 mg/kg dose wasn't cleared from the plasma until 50 h, and the 250 mg/kg dose had 20% of the initial dose retained in the plasma over the same time frame. Also, the volumes of distribution were quite high

(4 times the total blood volume of the mouse). This indicates a broad tissue distribution likely resulting from the amphiphilic nature of the PEO-PCL copolymer. Thus, the optimization of the block lengths of PEO for stability *in vitro* translated over to stability *in vivo* based on the pharmacokinetics and thermodynamic stability.

### 3.3.2 Stability of PEO-PCL micelles *in vivo*

Another approach to evaluating *in vivo* stability utilizes conjugated fluorescent dyes to $PEO_{45}$-$PCL_{21}$ copolymers.[82] A derivative of 5-fluorescein cadaverine (F-5-CADA) was conjugated to the terminal end of the PCL chain to investigate the stability of the $PEO_{45}$-$PCL_{21}$ micelles in PBS with 5% fetal bovine serum (FBS). F-5-CADA was in an inactivated form that could be hydrolytically activated. Through 48 h only 2% of the dye was fluorescent from the micelle solution. Thus these micelles were stable, because the intact micelles displayed virtually no fluorescence and the free dye emitted 33% fluorescence through 48 h and at the same conditions. The reason the free dye emitted only 33% is due to the slow partial hydrolytic activation in PBS. In RPMI-1640 cell culture medium with and without FBS, the micelles were less stable. Through 48 h in RPMI without serum, the PEO-PCL micelles had 13% activation, compared to 83% for free dye. The presence of serum drastically reduced the stability of the micelles as RPMI with 5% FBS, and FBS without RPMI activated 64% and 74% respectively. The free dye was fully activated in FBS without RPMI, while 83% of the free dye was activated in

RPMI with 5% FBS. These results demonstrate that FBS is an excellent screening agent for the stability of micelles since the equilibrium between micelles and unimers is media dependent. The stability of the micelles in FBS at 10 and 100 times the CMC depicted modest increases in stability (10-12%) with similar activations of F-5-CADA to those already mentioned. When these micelles were incubated with T24 human bladder carcinoma cells, an increase in fluorescence due to disruption of $PEO_{45}$-$PCL_{21}$-F-5-CADA micelles was seen with time in lysed cells and was substantial at 18 h (approx 70% of maximum). Intravenous bolus injections of $PEO_{45}$-$PCL_{21}$-F-5-CADA micelles showed accumulation in the bladder of hairless SKH-1 mice. This implies that the filtration in the kidneys disrupted these micelles. Both subcutaneous and intramuscular injections showed an increase in fluorescence signal at the site of injection after 1 h. These results suggest that subcutaneous and intramuscular injection provide a means for micelles to simply solubilize a drug and then be site-injected to a target organ or a solid tumor that is inoperable. Alternatively, if blanket coverage of the circulatory system needs to be to be treated as is the case in metastatic cancers, intravenous injection of the micelles would be the preferred route. Conjugated fluorescence dyes to PEO-PCL micelles *in vitro* and *in vivo* will play an important role in pre-clinical evaluation of micelle delivered drugs.

Though stability of the micelles *in vivo* is an important factor to consider, sometimes micelles need to protect the drug from degradation. This is the case with curcumin, a anti-cancer agent known to down regulate genes associated with angiogenesis of tumors.[83] PEO-PCL micelles with a PEO block length of 5 kDa

and PCL with a block length of 13 kDa were used to deliver curcumin to investigate the pharmacokinetics in Sprague-Dawley rats.[84] In this study, the micelle formulation of curcumin was compared to a solubilized formulation employing dimethylacetamide, soluble PEO with a block length of 0.4 kDa, and isotonic dextran. High performance liquid chromatography (HPLC) was used to analyze curcumin in plasma. Since curcumin is rapidly hydrolytically degraded, the soluble formulation had a half life of only a half an hour, while the PEO-PCL formulation increased this half life approximately 120 fold. After 2 h, HPLC wasn't able to detect curcumin from the soluble formulation, whereas curcumin from the PEO-PCL micelle formulation was detectable for 2 days. Not only did the PEO-PCL micelles protect curcumin from degradation, it also increased the solubility several orders of magnitude.

Another anticancer agent, 10-hydroxycamptothecin (HCPT), is a powerful antitumor drug against lung, ovarian, breast, and stomach cancers. Yet it also is limited therapeutically due to its poor water solubility and a short half-life *in vivo*. PEO-PCL micelles were used to encapsulate HCPT and were injected into Wistar rats and $S_{180}$ tumor bearing mice to evaluate the pharmacokinetics and tissue distribution.[73] A series of PEO-PCL micelles with variation in the hydrophilic PEO block length and lipophilic PCL block length were shown to have CMC's on the order of $10^{-7}$-$10^{-8}$ M. Radiolabeled $^{125}$I-HCPT depicted the largest tumor uptake over other organs in $S_{180}$ tumor bearing mice when a micelle with a PEO block length of

10 kDa was used. This micelle was compared against micelles with identical PCL block lengths and shells with 2 and 5 kDa PEO blocks. The half life of $^{125}$I-HCPT was also greatest in the micelle with the 10 kDa PEO block. Thus not only should the PCL block be optimized in preclinical studies, but so should the PEO block.

## 4.0 Conclusions

PEO-PCL micelles have many advantages over currently approved formulations (e.g. Cremophor® EL) for poorly water soluble drugs. They have been shown to be biocompatible with multiple cell lines, can provide extended release, and can retain a drugs biological activity *in vitro* and *in vivo*. One important factor in the design of future PEO-PCL cores is the achievement of the lowest possible CMC's (Table 1). This simple thermodynamic parameter provides much information on the intrinsic stability of these micelles. The use of FBS is an excellent accelerated stability agent to examine the CMC over time. In terms of straight drug delivery, increasing PCL block length with very poorly water soluble drugs usually increases encapsulation efficiency. This increase in encapsulation efficiency provides two measures: to reduce the cost to recover unloaded, very expensive drugs, and the potential to deliver higher doses as is often desired in the use of anti-cancer agents. However simply increasing the block length doesn't always increase drug encapsulation. In this case, it is useful to first understand the thermodynamics of interaction between the core and drug with the Flory-Huggins interaction parameter. If the core cannot be changed in some way to adjust this parameter, it is possible to

crosslink the core, or to use very lipophilic additives such as α-tocopherol. Increasing the cores number of chains relative to the corona through use of triblocks or star copolymers, though a viable alternative to diblocks, does not significantly improve the stability of these micelles or increase the encapsulation efficiency. These micelles stability does improve with increased PCL chain length or by adding substituent functional groups such as lipophilic groups (α-benzyl carboxylate) (Table 1). Additionally the CMC can be raised by adding hydrophilic functional groups such as carboxylic acids. Once the general micelle has been optimally designed for release, it is then relevant to evaluate the delivery vehicle *in vitro* and *in vivo*. A certain percentage of PEO-PCL micelles can enter cells through a combination of clathrin-mediated endocytosis and phagocytosis. Once the micelles have entered the cells they tend to localize in cytoplasmic organelles, specifically the Golgi apparatus and mitochondria. Though these implications of cellular entry and localization may not broadly affect the efficacy of these micelles as delivery systems, these points should be recognized if problems in the clinic arise. PEO-PCL micelles have been shown to enhance the pharmacokinetics of drugs *in vivo* through increasing their half life. This increase in half life is likely due to the protection of the drugs from hydrolytic degradation in the PCL core. These micelles can also protect the patient from unwanted hemolysis of red blood cells upon injection. During these *in vivo* studies, both block lengths should be optimized. In pre-clinical studies, the PCL block length should be optimized based on the drug release and encapsulation

efficiency. During *in vivo* studies, the PEO block should be optimized as it has shown the ability to increase the half life of some drugs. With the development of new chemistries in years to come, PCL core stability may be increased with new substituents, or new exotic geometries.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| A2780 | ovarian cancer cell line |
| atRA | all-trans retinoic acid |
| Bcl-2 (xL) | anti-apoptotic proteins encoded by an oncogene of the same name |
| CHO-K1 | chinese hamster ovary cell line |
| CMC | critical micelle concentration |
| CsA | cyclosporine A |
| DHT | dihydroxytestosterone |
| DLS | dynamic light scattering |
| DMEP | 4'-demethylepipodophyllotoxin |
| DSC | differential scanning calorimetry |
| DOX | doxorubicin |
| EGF | epidermal growth factor |
| F-5-CADA | 5-fluorescein cadaverine |
| FBS | fetal bovine serum |
| h | hour(s) |
| $^{3}H$ | Tritium |
| HCPT | 10-hydroxycamptothecin |
| Her2/neu | receptor tyrosine kinase at the cell membrane surface |
| Hsp90 | heat shock protein (90 kilo Daltons) |
| $^{125}I$ | radioiodine |
| $I_E$ | fluorescent emission of light from eximer of dipyrene at 480 nm |
| $I_M$ | fluorescent emission of light from monomer of dipyrene at 390 nm |
| $IC_{50}$ | half-maximal inhibitory concentration of cell death (in this context) |
| kDa | kilo Daltons |
| M | molar concentration (mols/liter) |
| MCF-7 | breast cancer cell line |
| MW | molecular weight |
| $N_{agg}$ | statistical-mechanics derived aggregation number |
| NIH 3T3 | National Institutes of Health fibroblast cell line |
| PEO | poly(ethylene oxide) |
| PBCL | poly(α-benzyl carboxylate ε-caprolactone) |
| PC 12 | rat neuroendocrine tumor cells |
| PCCL | poly(α-carboxylate ε-caprolactone) |
| PCL | poly(ε-caprolactone) |
| PSMA | prostate specific membrane antigen |
| PVL | poly(δ-valerolactone) |
| SANS | small angle neutron scattering |
| SAXS | small angle x-ray scattering |
| STAT3 | signal transducer and activator of transcription 3 |
| SKOV-3 | ovarian cancer cell line |

| | |
|---|---|
| SLS | static light scattering |
| $T_g$ | glass transition temperature |
| $T_m$ | melting temperature |
| Z | average-density aggregation number |