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# Nano-sized drug-loaded micelles deliver payload to lymph node immune cells and prolong allograft survival

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ABSTRACT

By delivering immunomodulatory drugs in vivo directly to lymph nodes draining an injection site, an opportunity exists to increase drug bioavailability to local immune cells. Importantly, particles smaller than 100 nm are efficiently transported through lymphatic vessels to draining lymph nodes. To investigate whether this approach could be used for local delivery of immunomodulatory drugs, amphiphilic poly (ethylene glycol)-*bl*-poly(propylene sulfide) (PEG-*bl*-PPS) block copolymers forming 50 nm micelles were used to encapsulate hydrophobic drugs. Micelle drainage was determined using fluorescent micelles and showed effective targeting of multiple immune cell subsets in lymph nodes. For functional studies of our formulations, two approaches were considered. To evaluate the efficacy of anti-inflammatory drug delivery, dendritic cell activation was shown to be prevented when mice were pretreated with micelles loaded with the glucocorticoid mometasone and then challenged with the TLR9 ligand, CpG. To evaluate whether immunosuppressive drug-loaded micelles were effective in prolonging MHC-mismatched allograft survival, BALB/c mice were treated for 14 consecutive days with drug-loaded micelles following transplantation of allogenic C57BL/6 tail skin. Micelles loaded with a mixture of rapamycin and tacrolimus prolonged allograft survival by 2-fold. Our results indicate that the drug-loaded micelle approach effectively targets the draining lymph nodes and exhibits proper immune regulation.



## 1. Introduction

By utilizing the lymphatics for particle drainage, drug loaded carriers have the opportunity to interact once with lymph node-resident immune cells directly before they reach the blood circulation, spleen, and liver [1]. Lymphatic transport has been shown to be in part dependent on particle size; following subcutaneous administration, nano-sized carriers less than 100 nm in diameter are taken up by lymphatic capillaries and target the draining lymph nodes [2,3]. The lymph nodes are major depots of immune cells such as T cells and dendritic cells (DCs) and are known to be key regulators in meditating the balance between tolerance and immunity. These immune cells also participate in the rejection or survival of allotransplants and thus are considered appropriate targets for influencing immune regulation following graft transplant [4,5]. Thus, lymph node-resident immune cells can be prime targets in a number of immunotherapeutic approaches.

Many immunotargeting approaches have focused on manipulating DCs ex vivo through the use of pharmacological mediators such as immunosuppressive or anti-inflammatory drugs [6]. These approaches are expensive and labor intensive and, as such, local delivery methods that regulate DCs or other immune cells including T cells could provide powerful alternatives. However, a major challenge for manipulating immune cells in vivo is the difficulty of delivering mediators to a targeted location in effective concentrations, since many of these drugs have low solubility. To address this problem, biodegradable polymer micelles 50 nm in size have been used to encapsulate insoluble, hydrophobic drugs in their core allowing for safe, aqueous delivery [7].

Hydrophobic drugs with immunomodulatory function including cyclosporin A and rapamycin (sirolimus) have been effectively encapsulated in micelles composed of amphiphilic poly(ethylene glycol) and poly(propylene sulfide) block copolymers (PEG-*bl*-PPS) [8,9]. PEG-*bl*-PPS micelles allow for sustained drug release, with a size that is compatible with our goal of lymphatic targeting for immune

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cell regulation in the draining lymph node [10]. Therefore, we sought to determine if drug-loaded micelles have similar trafficking and drainage in the lymphatic network in a mouse tail model as compared to PEG-based solid-core nanoparticles [3] and whether the drug activity is retained after administration. The mouse tail model has been extensively employed to investigate lymphatic trafficking because lymphatic flow is unidirectional towards the sciatic lymph nodes [11]. The kinetics of particle drainage can be evaluated by injecting fluorescent particles in the distal part of the mouse-tail and by examining cells in the draining lymph nodes by flow cytometry hours after injection. Using this method, we observed that fluorescent micelles were targeting multiple immune cell populations. Therefore, we assessed micelle formulations in separate models using insoluble drug types with different immunomodulatory capacity, namely either anti-inflammatory or immunosuppressive function.

As an example of anti-inflammatory delivery, the glucocorticoid drug mometasone furoate has been shown to reduce inflammation by decreasing the influx of immune cells and by activating the secretion of anti-inflammatory cytokines; however, mometasone use is normally limited to the treatment of asthma or inflammatory skin conditions such as eczema through non-parental routes [12]. Mometasone strongly effects bone marrow-derived DC maturation as shown by the prevention of the upregulation of cell surface activation markers [13] and thus is attractive for lymph node DC delivery. For therapies post transplantation, immunosuppressive rapamycin has been shown to inhibit mTOR and to act both on DCs as well as T cells [14]. However, rapamycin remains poorly soluble with only 15% bioavailability when delivered orally [15]. Another immunosuppressive with poor solubility, tacrolimus, has the same molecular target as cyclosporine A, inhibiting the activity of calcineurin, and has been shown to act synergistically with rapamycin to prolong transplant survival [16,17].

Thus, micelles encapsulating immunomodulatory drugs were evaluated for their ability to traffic to the lymph node and influence appropriate immune responses. Anti-inflammatory mometasone micelle formulations were evaluated for their ability to blunt maturation of dendritic cells in the lymph nodes after challenge with the TLR9 ligand, CpG, a DNA oligomer that mimics bacterial DNA sequences. To evaluate micelle formulations with an immunosuppressive mechanism of action, commonly utilized rapamycin and tacrolimus were encapsulated and assayed for their ability to prolong MHC-mismatched skin allograft transplant survival when the formulations were injected daily for 2 wk post-transplant. The results of these evaluations demonstrate that the PEG-*bl*-PPS micelle system is an effective drug delivery vehicle for both anti-inflammatory and immunosuppressive hydrophobic drugs capable of targeting lymph node resident immune cells.

## 2. Materials and methods

### 2.1. Micelle preparation and drug loading

PEG-*bl*-PPS block copolymers were synthesized by anionic ring opening polymerization of propylene sulfide, initiated by benzenethiol and terminated by addition of PEG-bromide, previously prepared as described [18,19]. Fluorescent PEG-*bl*-PPS-AF647 (AF647) were obtained by conjugation of fluorescent probes to $NH_2$-PPS-*bl*-PEG. In this case, the propylene sulfide was polymerized from the deprotection of a phthalimide bearing a thioacetate group using sodium methoxide. After polymerization of propylene sulfide, the block copolymer was formed by end capping with PEG-mesylate and the phthalimide group was hydrolized in ethanol with hydrazine hydrate heated to reflux (120 °C). Drugs were loaded into micelles using a cosolvent evaporation method [9]; 20 mg of polymer was dissolved in 0.3 mL dichloromethane (DCM) and 1 mL PBS was added dropwise to the solution under constant stirring at room temperature for 3 h, followed by 1 h of desiccation. For micelle drainage, 20 mg/mL PEG-*bl*-PPS-AF647 were loaded with 1 mg dexamethasone-fluorescein (Dex-FL, Invitrogen, Basel, Switzerland) in PBS. Micelles in a volume 500 μL were purified using a Sepharose 6B column in 0.5 mL fractions (Sigma, Buchs, Switzerland), and the fluorescence of the fractions were evaluated using a fluorimeter (Tecan Safire, Männedorf, Switzerland). As a comparison of size exclusion with free drug only, 0.2 mg Dex-FL was dissolved in acetone and then supplemented with 90% PBS until a final volume of 1 mL and purified as above. For other assays, micelles were loaded with the drugs mometasone furoate (Ivy Fine Chemicals, New Jersey, USA), rapamycin and tacrolimus FK-506 (LC Laboratories, Woburn, USA) with 10–20 mg/mL PEG-*bl*-PPS polymer. Unloaded drug was removed by centrifuging micelles for 1 min at 5000 *g* and drug-loaded micelles were sterilized through a 0.22 μm filter. Micelle size and polydispersity were measured by dynamic light scattering on a Zetasizer (Malvern, UK). To determine drug loading, micelles were freeze-dried, dissolved in THF, and loaded onto a Waters Styragel THF columns (Milford, MA, USA) for gel permeation chromatography (GPC), and loading was compared to injected standards of the drug dissolved in THF. Loading efficiencies were calculated based on the total drug encapsulated compared to the initial drug added. Drug retention inside micelles was evaluated by dialysis of 1 mL of micelles using 10,000 MWCO Spectrum cellulose membranes (VWR) into 2 L of water with constant stirring. Samples of 50 μL for GPC analysis were taken every 24 h, and the water sink was refreshed daily. To measure release of free drug from dialysis, rapamycin was dissolved in water at its solubility limit (approximately 2.5 μg/mL) and samples were taken and analyzed as above at 0, 2, and 24 h. Endotoxin levels were evaluated using HEK-Blue™ hTLR4 cells from InvivoGen (San Diego, CA, USA) as per manufacturer instructions. Soluble (non-micellar) drug preparations for injection of rapamycin and tacrolimus were made in 10% N,N-dimethylacetamide (Brunschwig, Basel, Switzerland) with 80% PEG400 (Hampton, Aliso Viejo, CA, USA) and 10% Tween80 (Sigma) similar to reported methods [20]. To determine hydrophobicity, average logP values of drugs were measured using the ALOGPS 2.1 program [21].

### 2.2. Animals

BALB/c and C57BL/6 mice were purchased from Charles River (L'Arbresle Cedex, France) and used when 6–8 weeks old. All animals were housed in the conventional facility of EPFL and utilized in accordance with the Veterinary Authorities of the Canton Vaud under Swiss law.

### 2.3. Micelle drainage studies

BALB/c mice were injected intradermally twice in the distal portion of the tail with 15 μL of 10 mg/mL PEG-PPS-AF647 micelles loaded with 0.5 mg/mL dexamethasone-fluorescein. Mice were sacrificed at 2, 12, and 24 h after injection, and the draining sciatic and subiliac lymph nodes were harvested. Single cell suspensions were made after digesting lymph nodes in 1 mg/mL collagenase D (Roche, Mannheim, Germany) for 30 min at 37 °C and passing through a 70 μm cell sieve. Cells were stained with LIVE/DEAD Fixable Red Dead Cell Stain (Invitrogen) and then for surface markers (anti-mouse F4/80-PE, anti-mouse B220-PerCPCy5.5, anti-mouse CD11b-PECy7, anti-mouse CD3-Pacific Blue, and anti-mouse CD11c-APC-eFluor780; eBioscience, San Diego, CA). Samples were evaluated by flow cytometry (Dako CyAn ADP, Beckman Coulter, Brea, CA, USA) and data analyzed with FlowJo (TreeStar, Ashland, Oregon, USA). For determining cell subsets, live cells positive for AF647 micelles were further subdivided into immune subtypes based on surface marker expression: T cells (CD3+), B cells (B220+), DCs (CD11c+ CD11b+), and macrophages (F4/80+ CD11b+).