This is an Accepted Manuscript, which has been through the RSC Publishing peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, which is prior to technical editing, formatting and proof reading. This free service from RSC Publishing allows authors to make their results available to the community, in citable form, before publication of the edited article. This Accepted Manuscript will be replaced by the edited and formatted Advance Article as soon as this is available.

To cite this manuscript please use its permanent Digital Object Identifier (DOI®), which is identical for all formats of publication.

More information about Accepted Manuscripts can be found in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics contained in the manuscript submitted by the author(s) which may alter content, and that the standard Terms & Conditions and the ethical guidelines that apply to the journal are still applicable. In no event shall the RSC be held responsible for any errors or omissions in these Accepted Manuscript manuscripts or any consequences arising from the use of any information contained in them.
Hybrid Polymersomes: Facile Manipulation of Vesicular Surface for Enhancing Cellular Interaction†

Xingfeng Su, a Shaqireen Kwajah Mohamed Moinuddeen, b Lucia Mori,* b and Madhavan Nallani* a,c

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Facile surface modification via the blending of lipids and block-co-polymer to assemble hybrid vesicles was investigated for improving cellular interaction and antigen delivery of poly(ethylene glycol) (PEG)-based polymersomes. Cationic lipids (DOTAP) incorporated into PEG-b-PBD polymersomes increased binding and uptake of vesicles by antigen-presenting cells, while preserving the stability and biocompatibility of PEG-based polymersomes, resulting in enhanced cellular delivery of a loaded lipid antigen sulfatide.

Polymeromes are vesicles formed by the self-assembly of amphiphilic block-copolymers in aqueous environment. Structurally, they resemble liposomes but exhibit greater membrane stability due to the higher molecular weight and lower critical aggregation concentration of their constituent amphiphiles. 1,2 With respect to liposomes, polymersomes are able to enclose larger quantities of hydrophobic compounds due to the increased membrane thickness and stability, while retaining the capacity to load both hydrophilic and hydrophobic molecules into the aqueous core and hydrophobic membrane, respectively.3 These beneficial properties make them highly attractive as delivery vehicles for biomedical applications, which has seen a surge in research interest in recent years.3–5 The vast majority of polymersomes reported for use as therapeutic carriers are assembled from diblock copolymers with poly(ethylene glycol) (PEG) as the hydrophilic block. This component creates a relatively inert, brush-like outer shell, which provides steric stabilization and confers “stealth” properties to the vesicles, allowing them to repel protein adsorption and avoid clearance by the reticuloendothelial system, thereby prolonging circulation times.5 While the hydrophilic PEG shell imparts excellent biocompatibility, it also diminishes the uptake of these carriers by cells since the PEG brush interferes with cell-cell interactions. This effect was demonstrated by Discher et al., where PEG–poly(butadiene) (PEG-PBD) polymersomes incubated with phagocytes fail to show any adhesion or invoke any cellular response.6 In addition, it has been shown that the presence of PEG on carrier surfaces can inhibit ligand-mediated targeting of carriers, due to steric effects.6,7

To overcome these limitations imposed by the PEGylated surface, alternative polymers have been explored as the hydrophilic block. For example, a zwitterionic, phospholipid mimic, PMPC that resembles phosphorylcholine (PC) found in cell membranes, has been shown to form polymersomes that exhibit increased cellular interaction due to the enhanced affinity of PMPC with cell surface.8 Instead of synthesizing a cell membrane mimic, another approach is to modulate polymersome membrane properties by physically blending phospholipids and block copolymers to engineer hybrid vesicles.8–10 In the case of PEG-PBD polymersomes, it has been reported that the lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), a major constituent of physiological cell membranes, forms homogeneous membranes when mixed with the block-copolymer.11 Despite the clear advantages of combining the desirable characteristics of both constituents into one system, few studies to date have explored phospholipid/copolymer hybrid vesicles for biological applications. Nonetheless, in a recent study, Cheng et al. reported the tuning of PEG-PBD polymersome membrane by mixing the block copolymers with 10 – 25 % of lipid (hydrogenated soy phosphatidylcholine (HSPC)), as well as a PEG-ylated lipid functionalized with either a folate group or an amino-group for conjugation to an antibody, for targeting cancer cells.12 These ligand-functionalized hybrid vesicles led to significant enhancement in cellular-vesicle interactions in in vitro cell studies and in vivo murine tumor models, due to more efficient ligand-binding by diluting the PEG shell with lipids. The aim of this study was to investigate whether the blending of a cationic phospholipid by introducing positive charges in the polymersome membrane, could enhance the interactions of PEG-PBD polymersomes and consequently the delivery of a lipid antigen to antigen-presenting cells (APCs) without the need of synthesis and conjugation of any explicit targeting ligands. Targeting moieties do not act as a ‘homing beacon’ to the desired target cells. Instead, targeted carriers must first come into close contact with target cells for ligand-binding to mediate targeted cellular uptake and delivery. Generally, positively-charged carriers are not only more thermodynamically stable but also exhibit increased interaction with cell membranes as compared to non-charged carriers, owing to electrostatic attraction with negatively-charged cell membrane components.13 This has been reported for polymersomes where the attachment of Tat peptide, a cationic cell-penetrating peptide to PEG-PBD polymersomes was shown to increase dendritic cell uptake in vitro.14 However, as with most functionalized polymersomes, the modification of vesicle surface usually entails either the conjugation of functional ligands to preformed vesicles or the self-assembly of end-group functionalized block copolymers where the parent block-copolymer has to be first chemically modified.15 Such an approach is not always trivial as difficulties may arise from purification and chemical characterization following conjugation. In contrast, the mixing of the pristine block-copolymer with commercially available, well-characterized phospholipids bearing appropriate functional groups is simple and also offers the ease of tuning the density of functionalities on vesicle surface via its composition to achieve desired membrane properties. Here, we
report the assembly of stable polymersomes with hybrid membranes, composed of PEG-PBD and 1,2-dioleoyl-3-(trimethylammonium)propane (DOTAP) and demonstrate their ability to mediate enhanced cellular interactions and delivery in 2 types of human APCs, THP-1 monocytes and C1R B cells.

PEG-PBD polymersomes and lipid-containing hybrid polymersomes were formed by drying the block-copolymer and DOTAP (25 mol%) to make a film and rehydrating in PBS (0.1 mM phosphate, pH 7.4) for self-assembly into vesicles. The vesicles were labeled by adding 1 mol% rhodamine-PE (phosphatidylethanolamine) where such amphiphilic dye molecules have been shown in previous studies to integrate within the vesicle membranes and thereby enable direct visualization of vesicles interaction with cells via confocal microscopy.\(^{19-21}\) The resulting vesicles were extruded to tune their size to ~200 nm, the size limit reported for uptake via endocytosis by APCs.\(^{22}\) To purify the vesicles, the suspension was subjected to dialysis and the removal of free dye molecules confirmed via centrifugal filtration using filters with 1 MDa size cutoff (see supplementary information). Characterizing the dialysed vesicles via dynamic light scattering (DLS), both pure and hybrid polymersomes have a hydrodynamic diameter of ~230 nm and a polydispersity index of 0.1-0.2. The vesicles were stable over 1 month-time at room temperature with no sign of turbidity/sedimentation and no detectable aggregation. Their stability was further collaborated by DLS, where minimal change in average hydrodynamic diameter, polydispersity index and count rate was observed for at least 1 month for both PEG-PBD and hybrid polymersomes. (Fig. 1) This property is in agreement with and even extends the previous study by Cheng et al. where PEG-PBD vesicles prepared with up to 25 mol% HSPC did not exhibit any significant change in hydrodynamic diameter over 10 days.\(^{15}\)

![Fig. 1](image1.png)

**Fig. 1** Stability of PEG-PBD polymersomes (open points and dotted lines) and DOTAP-PEG-PBD hybrid polymersome (solid points and lines) dispersions at room temperature in PBS measured by DLS. 5 measurement runs were performed on 3 samples at each time point: mean diameter (squares), count rate (triangles) and polydispersity index (circles). Bars represent standard deviations of triplicate samples.

![Fig. 2](image2.png)

**Fig. 2** Hybrid DOTAP-PEG-PBD polymersomes enhance cellular binding and internalization in APCs. Representative confocal microscopy images of C1R and THP-1 cells incubated for 4 and 24 h at 37 °C with rhodamine-PE labeled PEG-PBD or hybrid DOTAP-PEG-PBD polymersomes (red). C1R cells incubated with hybrid DOTAP polymersomes (a), C1R cells incubated with PEG-PBD polymersomes (b), THP-1 cells with hybrid DOTAP polymersomes (c) and THP-1 cells with PEG-PBD polymersomes (d). Scale bars 20 µm.

We evaluated the cellular interactions of hybrid polymersomes with human cells of hematopoietic origin that play a pivotal role in immune responses, and could be therefore important targets for delivery of therapeutics for conditions ranging from infections, inflammation, auto-immune diseases and cancer.\(^{23,24}\) We selected cells with well-known APC function: THP-1, a monocytic cell line of myeloid origin and C1R, a B lymphoblastoid cell line of lymphoid origin. To assess the capability of hybrid polymersomes to facilitate cell binding and uptake, vesicles were incubated with THP-1 and C1R cells, following which the cells were washed to remove unbound vesicles and observed under a
confocal microscope. After 4 h incubation, we detected significantly higher binding of DOTAP-bearing hybrid vesicles to C1R cell surface (Fig. 2a) and increased internalization in THP-1 (Fig. 2c) compared to unmodified PEG-PBD polymersomes (Fig. 2b and 2d for C1R and THP-1 cells respectively). Even by extending incubation to 24 h, cellular binding and uptake of PEG-PBD polymersomes remained low, while hybrid vesicles show even greater interaction. The different localization of hybrid polymersomes in C1R and THP-1 cells is not surprising given that B cells are known to display lower endocytosis/phagocytosis activities relative to monocytes. Further, to demonstrate that the cationic charges introduced by DOTAP into vesicle membranes were responsible for the improved cellular interaction, we compared hybrid polymersomes prepared by blending 25 mol% zwitterionic POPC with those blended with cationic DOTAP, where vesicle uptake by THP-1 cells resembled that of unmodified PEG-PBD vesicles (supplementary Fig. 1). In all these studies, the viability of cells before and after treatment with vesicles was always above 90% as determined by trypan blue exclusion (supplementary Fig. 2), with no significant differences found between unmodified PEG-PBD and hybrid polymersomes, indicating that the addition of a cationic lipid at 25 mol% did not impact the biocompatibility of vesicles while effecting an enhancement in cellular interactions.

Next, we explored the potential of DOTAP-modified hybrid polymersomes as nanocarriers to deliver lipid molecules of particular interest. Sulfatides (SF), major components of myelin sheaths, are lipid self-antigen targets of autoreactive cells during multiple sclerosis (MS). A specific immune intervention to revert autoimmunity represents an unmet clinical need. To elicit an antigen-specific T cell response, the lipid antigen must first be delivered into the endolysosomal compartments of APCs where antigen loading into dedicated lipid-presenting molecules of the CD1 family takes place. For effective delivery, lipid antigens in particular, due to their poor solubility in aqueous environments, would stand to benefit from formulation into delivery vehicles. However, the use of drug carriers to facilitate the delivery of glycolipid antigens has thus far received relatively little attention and attempts have mainly been based on liposomal formulations where the unstable nature of liposomes remains an issue. As described earlier, this limitation can be addressed by the use of polymersomes whose superior stability warrants an edge over liposomes. As a first step towards the development of lipid antigen-coupled carriers as tools for specific immune intervention, we blended 1 mol% of SF labeled with a NBD dye (NBD-SF) into hybrid DOTAP-PEG-PBD polymersomes and tracked their intracellular trafficking following delivery into THP-1 cells. As expected with amphiphilic cargo molecules, NBD-SF was readily loaded into vesicles. A loading efficiency of ~90% was estimated by fluorescence measurements on post-dialysis suspensions and the removal of free SF confirmed via centrifugal filtration as above. (supplementary Fig. 3) NBD-SF was also endocytosed efficiently by THP-1 cells as observed previously (Fig. 3a,b). In contrast, delivery of NBD-SF at similar concentrations alone, in pure or POPC hybrid vesicles (similar SF loading efficiencies as DOTAP hybrid vesicles) all resulted in negligible SF uptake following a 24 h incubation (supplementary Fig. 4), confirming that formulation into hybrid DOTAP-polymersomes enhanced SF delivery to cells in vitro. To determine the intracellular localization of SF delivered by hybrid DOTAP-polymersomes, the endolysosomal compartments were stained with either Alexa Fluor 633-labeled transferrin or an APC-labeled anti-LAMP1 antibody to identify early/recycling endosomes and late endosomes/lysosomes, respectively. We detected colocalization of NBD-SF with both markers (Fig. 3e,f), suggesting that the lipid antigen was transported into the appropriate cellular compartments for potential loading onto CD1 molecules, a pre-requisite for eliciting lipid-specific immune responses.

In summary, we have demonstrated a strategy of blending cationic phospholipids with block-copolymers during vesicle assembly to overcome the existing limitation of poor cellular interaction exhibited by PEG-based polymersomes. Consequently, enhanced delivery of loaded lipid self-antigens, sulfatides into endolysosomes was achieved by hybrid DOTAP-polymersomes in a model APC, THP-1 cells. We are currently investigating the stimulatory capacity of sulfatides delivered by hybrid DOTAP vesicles using sulfatide-specific T cells in vitro. In addition, further improvement can be considered such as the use of degradable block-copolymer for constructing fully biodegradable carriers able to modulate cargo release. The potential of utilizing hybrid polymersomes as antigen carriers may have important implications for the development of effective lipid-based immunotherapies.

**Acknowledgements**
We thank Hans-Peter de Hoog (IMRE, A*STAR) for discussion. We acknowledge the Joint Council Office, IMRE and StgN, A*STAR for financial support.

Notes and references