Multivalent polymer vesicles *via* surface functionalization†

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A new method was developed for the conjugation of multivalent dendritic groups to polymer vesicle surfaces.

Amphiphilic block copolymers have been shown to assemble into a vast array of interesting morphologies ranging from spherical micelles[1] to helical rods,[2] toroids,[3] vesicles,[4,5] macroscopic tubes,[6] and multicompartment cylinders.[7] Polymer vesicles in particular have gained significant attention in recent years, likely due to their structural similarity to phospholipid vesicles. Phospholipid vesicles are important components of biological systems where they serve as cell membranes, and they have also been extensively investigated in areas such as gene and drug delivery.[8] In comparison with phospholipid vesicles, some polymer vesicles have been shown to have properties such as increased strength and decreased permeability, which may be advantageous for their application as biomaterials.[9] Indeed, reports on the use of polymer vesicles as carriers of proteins,[10,11] hydrophilic drugs,[12,13] and optical imaging agents[14] are emerging.

In the same manner that cell surface groups are critical in determining the interactions between cells and their responses to environmental stimuli, the surface functionalities of polymer vesicles will also determine important properties including biodistribution, cellular uptake, and targeting. However, relatively little work has focused on introducing functional groups to polymer vesicle surfaces. Thus far, biotin,[15,16] peptides[17] and proteins[16,18] have been conjugated by modifications of single polymer terminal groups. However, much effort is still needed to control vesicle properties through surface functionalization.

Here we present a method for introducing well-defined dendrons to polymer vesicles. This approach to surface functionalization may result in several significant advantages. For example, dendrons are multivalent, as many individual small molecules can be conjugated to their peripheries. Multivalent interactions are prevalent in biological systems and much research has shown that molecules such as carbohydrates and peptides exhibit increased binding strength when presented in a multivalent manner.[19] In addition, the branched architecture of the dendron makes it unlikely that it is buried within the vesicle membrane. Therefore, in comparison with individual small molecules conjugated to linear polymer termini, the dendron's peripheral groups should be readily available on the vesicle surface for interaction with biological targets.

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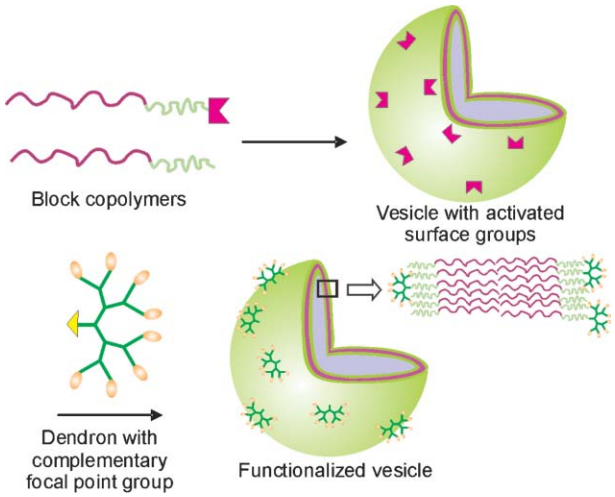
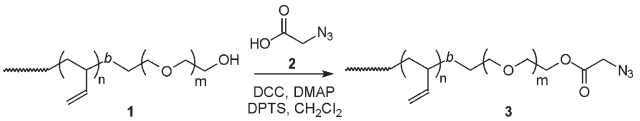


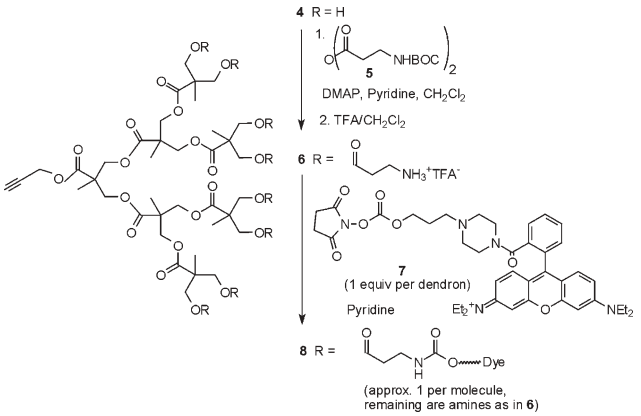
Fig. 1 Schematic for vesicle functionalization with dendritic groups.

Two general approaches can be envisioned for the incorporation of dendrons onto polymer vesicle surfaces. One approach involves the synthesis of amphiphilic polymers having terminal dendritic groups, followed by their assembly into vesicles. Another approach involves the assembly of vesicles having "activated" surface groups, followed by their reaction with dendrons having complementary groups at their focal points. The latter approach is described here and is illustrated in Fig. 1. This is the first method that results in polymer vesicles formed from mixtures of amphiphiles having different architectures.

To illustrate the new strategy, initial work has been carried out using the amphiphilic linear diblock copolymer poly(butadiene-*b*-ethylene oxide) (PBD–PEO) with a composition of 6500 g mol^{-1} PBD (>80% 1,2-addition) and 3900 g mol^{-1} PEO. Vesicles formed using closely related polymers have been extensively investigated and found to be highly stable and biocompatible.[9,20,21] As shown in Scheme 1, a terminal azide group was introduced to PBD–PEO (**1**) by reaction of the terminal hydroxyl with azidoacetic acid (**2**) to provide PBD–PEO–N_3 (**3**). The terminal azide, which should be presented on the vesicle surface, allows for the conjugation of a dendron with an alkyne focal point by a copper(I) catalyzed 3 + 2 "click" cycloaddition.[22] This reaction is highly efficient, even in cases involving substantial steric hindrance,[23] and importantly it



Scheme 1 Synthesis of PBD–PEO–N_3 (**3**).



Scheme 2 Synthesis of dye-labelled dendron (**8**).

can be carried out in water. It has been successfully applied to phospholipid vesicles[24] and very recently to polymer vesicles.[16] To ensure that the introduction of the azide terminal group did not disrupt the assembly of the polymers, vesicles were prepared by hydration of a thin film of **3** in the presence of the hydrophobic dye Nile Red. Confocal laser scanning microscopy (CLSM) was used to verify the presence of micron-sized vesicles (see ESI†).

A polyester dendron based on 2,2-bis(hydroxymethyl)propionic acid was selected due to its ease of synthesis and biocompatibility.[25] The alkyne functionalized dendron **4** was prepared as previously reported.[26] Unfortunately, this dendron has peripheral hydroxyl groups, which are not ideal functional handles for further derivatization with chromophores and ligands of biological interest. Therefore, the peripheral groups were converted to amines by reaction with anhydride **5**, followed by deprotection to provide dendron **6** (Scheme 2). Dendron **6** was then reacted with the rhodamine derivative **7**[27] to provide **8** with approximately one chromophore per dendron statistically. The extinction coefficient (ε) for **8** was measured in $CHCl_3$–MeOH. The dye provides a probe that can be used to monitor the conjugation of **8** to the vesicle surface.

The conjugation of an individual small molecule to the terminus of a linear polymer does not alter the polymer architecture, but the conjugation of dendron **8** to **3** results in a new polymer with a significantly different linear-dendritic architecture. Unlike linear polymers **1** and **3**, a linear-dendritic polymer is not expected to favour vesicle formation due to unfavourable steric interactions between the bulky dendritic end groups in the assembly. Therefore, it was of particular interest to determine the percentage of linear-dendritic polymer that could be incorporated into vesicles while still retaining their morphology.

In order to control the percentage of polymers that could be functionalized with **8**, and thus the percentage of linear-dendritic polymers in the resulting vesicles, mixtures of **1** and **3** ranging from 0–100% **3** were prepared (Table 1). Vesicle formation was carried out as described above, except that Nile Red was not used. Prior to the click reaction the vesicles were sonicated for 30 min to ensure that they were well dispersed. Click reactions were carried out over 24 h using 1 mM $CuSO_4$, 25 mM sodium ascorbate, 2.3 mM bathophenanthrolinedisulfonic acid and 4 equivalents of **8** with respect to **3**, then excess dendron was removed by dialysis.

The resulting assemblies were imaged by CLSM. At lower concentrations of **3**, up to about 20%, well dispersed fluorescent vesicles were observed, as shown in Fig. 2a. As dendron **8** is the only source of fluorescence, this verifies its successful conjugation to the vesicle surface. At higher concentrations of **3**, such as 40% and above, vesicles were observed but there was a significant degree of aggregation (Fig. 2b). This indicates that the vesicles are somewhat destabilized when too much linear-dendritic polymer is incorporated.

Table 1 Surface functionalization results[a]

Vesicles	% **3**	% Linear-dendritic polymer in resulting vesicles	% Yield of conjugation
9a	0	None detected	NA
9b	1	0.65	65
9c	2	1.36	68
9d	5	2.96	59
9e	7	4.49	64
9f	10	6.32 ± 0.25	63 ± 3
9g	20	13.8	69
9h	40	16.5	41
9i	70	18.8	27
9j	100	20.8	21

[a] Vesicles are composed of polymer **1** with varying percentages of **3**.

After removal of water, the amount of conjugated dendron was measured by UV-visible spectroscopy in $CHCl_3$–MeOH. A comparison of the percentage of **3** used in the vesicle preparation *versus* the resulting percentage of linear-dendritic polymers is given

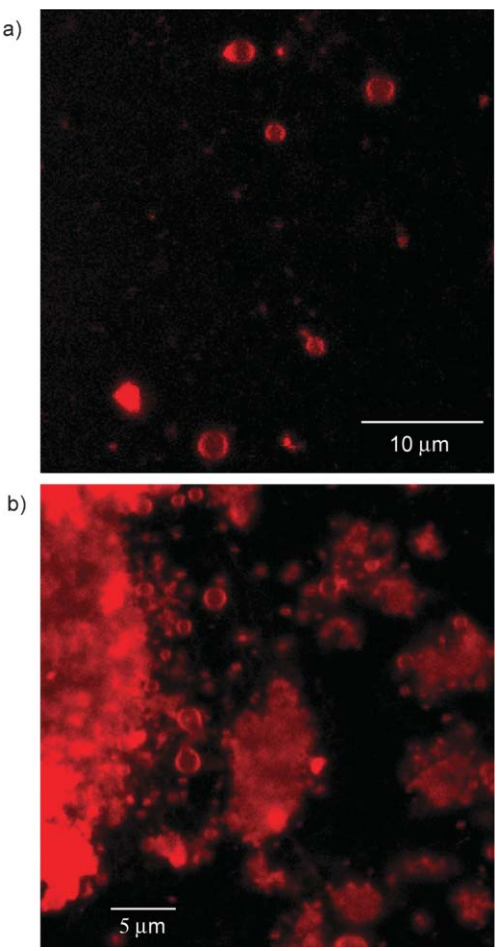


Fig. 2 CLSM images of vesicles (a) **9d** and (b) **9i**.

in Table 1. At low percentages of **3** (**9b**–**9g**), the yields are consistently quite high (60–70%). In fact, as close to 50% of **3** may be inaccessible on the interior of the vesicles, the yields are even higher than anticipated. It is probable that some interior azides move to the surface during the 24 h course of the reaction, and are subsequently functionalized. As the percentage of **3** increases beyond 20% (**9h**–**9j**), the yields drop dramatically, likely due to steric hindrance at the vesicle surface which prohibits the conjugation of additional dendrons. To examine the reproducibility of the functionalization, vesicles containing 10% **3** (**9f**) were subjected three times to identical conjugation conditions. The standard deviation was found to be <4%. It is also notable that when vesicles containing no **3** (**9a**) were subjected to the procedure above (same quantity of dendron **8** as for **9b**), no absorbance was detected in the resulting product, verifying that non-conjugated dendron is effectively removed by the dialysis. At higher percentages of **3**, conjugated dendron could also be detected by ^{1}H NMR spectroscopy, but due to the intensity of the polymer peaks relative to the dendron peaks and the overlap of key peaks, this measurement was not quantitative.

Finally, the reconstitution of functionalized vesicles in water following evaporation from $CHCl_3$–MeOH was investigated. This was of interest to further study the stability of the vesicles and to explore the possibility of preparing vesicles directly from pre-synthesized linear-dendritic amphiphiles. Indeed, functionalized vesicles resulting from lower concentrations of **3** (<20%) were successfully reconstituted by sonication of a thin film of the polymer in water. Higher concentrations of **3** led to aggregates.

In conclusion, a method for the conjugation of multivalent dendritic scaffolds to polymer vesicle surfaces was developed. The effect of this surface modification on the vesicle morphology and stability was investigated. Based on conjugation yields, CLSM images, and attempts at vesicle reconstitution, the ideal percentage of azide-functionalized polymer to achieve good surface coverage without risking vesicle destabilization following dendron conjugation is 10–20%. It is anticipated that this approach will be highly versatile as the peripheral amine groups of the dendron can be easily functionalized with carboxylic acid, NHS-ester, or isothiocyanate derivatives of biological ligands either prior to or after the click reactions. In addition, the approach should be readily adapted to vesicles formed from a variety of different polymer amphiphiles. The development of effective surface functionalization techniques has the capacity to significantly expand biomedical applications of polymer vesicles by providing a means to control their biodistribution, cell uptake, and target specificity.

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