Delivery Platforms

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Multifunctional Trackable Dendritic Scaffolds and Delivery Agents**

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Dendrimers and other three-dimensional molecular assemblies are attractive scaffolds for biological delivery agents and diagnostic probes[1,2] due to their globular shape, modular structure, monodispersity, and plurality of functional end groups.[3] To address this potential, a number of strategies and related dendritic architectures have been developed for delivery of bioactive molecules to desired cells or tissue,[4] with encapsulation[5] and covalent attachment to the dendritic chain ends being two major approaches.[6] While the encapsulation of drugs or dyes within the inner cavities of the dendrimer is promising,[5] in most cases only a limited number of guest molecules can be encapsulated even with dendrimers of high generations.[4d] Moreover, the noncovalent nature of the encapsulation makes it a challenge to control the stability of the loaded carrier and subsequent release of the payload.[4e] An alternative strategy exploits the large number of dendritic chain ends to carry the cargo molecules.[6] However, loading of large amounts of hydrophobic drugs or dyes can alter the dendrimer surface properties and decrease its solubility and bio-compatibility.[7] Partial functionalization[8] alleviates this issue but results in random chain-end modification leading to a dispersity in loading, variable bio-performance, and in many cases only low degrees of surface functionalization can be achieved without significantly changing the surface properties.[9]

In order to maintain both high loading and monodispersity, an alternative design is to covalently attach the cargo molecules to the interior of the dendrimer.[10] This strategy overcomes the challenges associated with surface functionalization, allowing high and reproducible loading without significantly altering the surface properties of the dendritic scaffold. To illustrate the power of this novel strategy, we report an accelerated synthesis of orthogonal surface and internally functionalized dendrimers[11] and their application as multifunctional dendritic scaffolds (Figure 1). As model

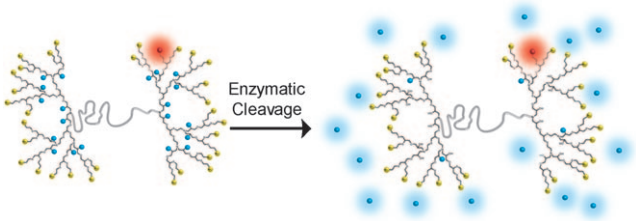

Figure 1. Modular design of dendritic scaffolds with protonated amino groups at the chain ends for cellular uptake (yellow). Upon enzymatic cleavage, the covalently attached, internal "blue" dyes are released while the noncleavable "red" dye allows for monitoring of the dendrimer itself.

delivery and diagnostic units, two different dyes were conjugated to the dendrimer: multiple coumarin units (blue in Figure 1) were loaded internally through a cleavable linker and a single Alexa647 dye (red in Figure 1) was conjugated to the surface through a stable amide bond. This dual labeling allows the dendritic scaffold and the model payload to be individually tracked in living cells at the same time. An additional facet of the platform design is the presence of numerous protonated amino groups at the chain ends of the dendritic fragments (yellow in Figure 1), which are designed to induce cell internalization through endocytosis, and while cationic materials may be toxic, they serve as a useful model system. Once inside the cell, it is envisaged that hydrolytic enzymes would cleave the internal coumarin units from the scaffold, resulting in release of the model coumarin payload and appearance of fluorescence.

The numerous structural requirements for these multifunctional dendritic scaffolds necessitated the development of an alternating sequence of "amine–epoxy" and "thiol–yne" coupling reactions (Scheme 1). In contrast to traditional thiol–ene chemistry used previously,[11] thiol–yne coupling leads to the addition of two thiols across the triple bond which, when combined with amine–epoxy chemistry, results in accelerated generation growth at each step of the synthesis (an AB_2/CD_2 approach)[12a] with the internal hydroxy groups being carried through the synthesis without the need for protection/deprotection steps.

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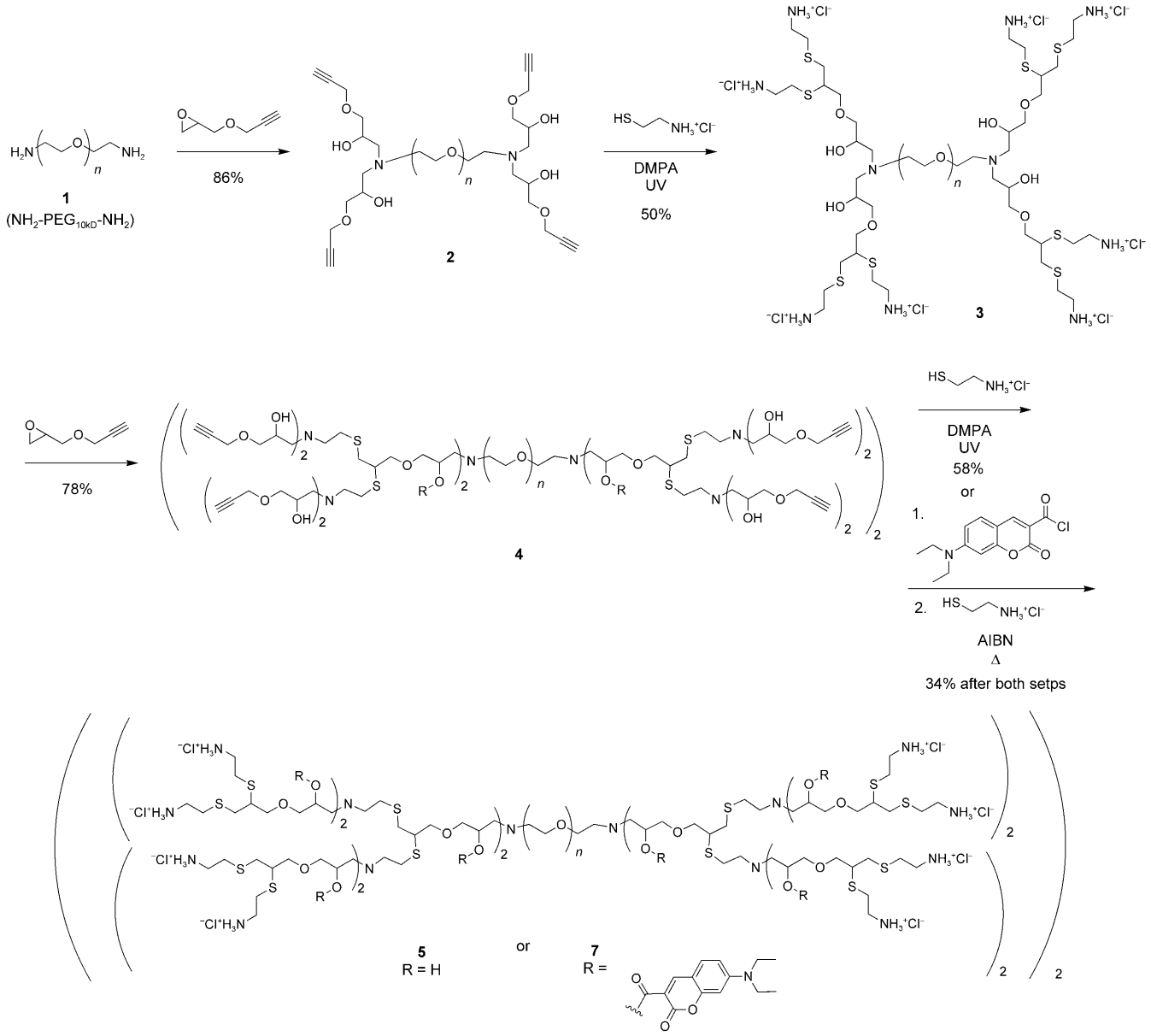


Scheme 1. Synthetic strategy for the synthesis of internally functionalized, hybrid dendritic macromolecule, [G-4]-(coumarin)$_{20}$ (**7**), based on a 10 kDa polyethylene glycol (PEG) core. DMPA = 2,2-dimethoxy-2-phenylacetophenone, AIBN = azobisisobutyronitrile.

The starting material was a 10 kDa bis-amine chain-end terminated polyethylene glycol (PEG), **1**, which functions as a core[12b] to enhance water solubility of the dendritic scaffold while also serving as a soluble support, simplifying purification of these hybrid dendritic–linear macromolecules.[13] The initial reaction involves the addition of propargyl glycidyl ether, to the PEG-diamine **1** to give the tetra-alkyne **2** which on thiol–yne coupling[14] with cysteamine hydrochloride gives the second-generation octaamine **3**. Repetition of this two-step procedure then gives the corresponding fourth-generation macromolecule **5** which contains 20 internal hydroxy groups and 32 primary amino groups at the chain ends. Alternatively, functionalization of the internal hydroxy groups was performed at the terminal alkyne stage of the synthesis (i.e. **4**) through facile esterification with an excess of 7-(diethylamino)coumarin-3-carbonyl chloride. The internally functionalized derivative, **6**, was dialyzed to remove excess coumarin, followed by thermal thiol–yne coupling with cysteamine hydrochloride in the presence of AIBN to give the hybrid dendritic structure, [G-4]-(coumarin)$_{20}$ (**7**), which was shown by ^{1}H NMR spectroscopy to contain ca. 20 coumarin units attached to the internal hydroxy groups. A combination of NMR spectroscopy, MALDI mass spectroscopy and GPC allowed full structural characterization of these novel functionalized, hybrid dendritic–linear macromolecules.

The core–shell structure of the labeled dendritic–linear macromolecules was initially examined by fluorescence spectroscopy, which showed significant quenching for the dendrimer-bonded coumarin (Figure 2a) when compared to the corresponding small molecule. This quenching of fluores-

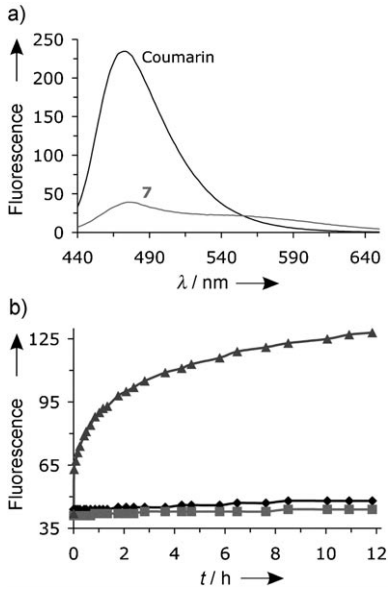
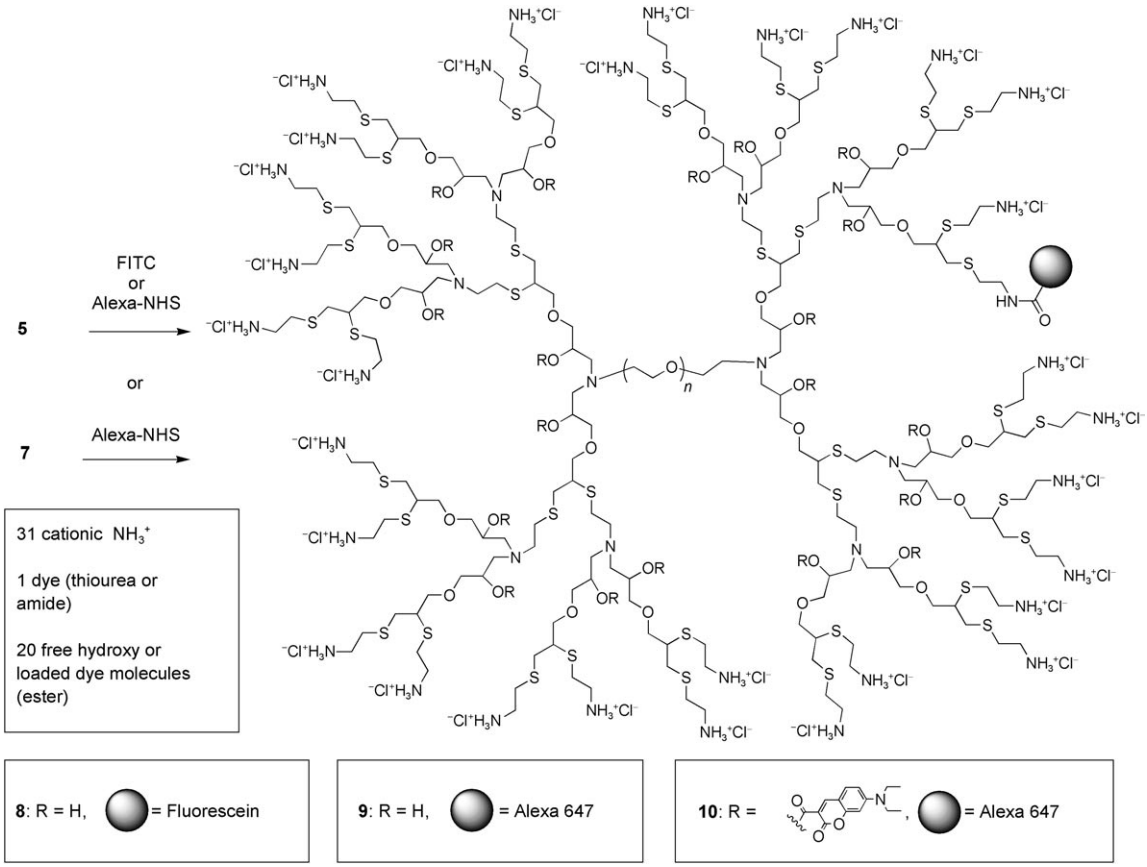


Figure 2. a) Fluorescence spectra of free coumarin and [G-4]-(coumarin)$_{20}$ (**7**). b) Fluorescence intensity of **7** at 480 nm as a function of time in the presence of esterase at pH 7.4 (▲) and in the absence of esterase at pH 5.0 (■) and 7.4 (◆).

cence is due to the high local concentration of coumarin groups attached to the interior of the dendrimer[6a,15] and on release of the model coumarin payload, the fluorescence should be restored. To demonstrate that the covalently attached dyes inside the dendrimer are accessible to enzymes, and therefore susceptible to enzymatic cleavage, functionalized dendrimer **7** was incubated with porcine liver esterase (PLE).[16] A kinetic plot of the maximum intensity at 480 nm as a function of time is presented in Figure 2b and shows a significant increase in fluorescence only in the presence of PLE.

This implies that PLE can access and hydrolyze the internal ester bonds connecting the coumarin units to the dendritic scaffold with only minor cleavage being observed at pH 5.0 or 7.4. The cleavage of the internal coumarin units were also confirmed by HPLC experiments which showed release only in the presence of esterase (see Supporting Information). The dequenching of the coumarin dye after release is of crucial importance for the cell studies as it provides insight into ester bond cleavage inside living cells while also allowing independent tracking of the payload and the scaffold.

Having demonstrated the increase in fluorescence on payload release, a series of model compounds were prepared for in vitro experiments and to examine the internalization of these hybrid structures into living cells.[17] For tracking of the dendritic scaffold, the amino-functionalized dendrimer **5** was conjugated with an average of one fluorescein isothiocyanate (FITC) dye to yield the labeled fourth-generation dendrimer, **8** (Scheme 2). Alternatively, Alexa647 was coupled with the dendrimers **5** and **7** to yield the labeled derivatives [G-4]-



Scheme 2. Synthesis of labeled dendrimers [G-4]-FITC (**8**), [G-4]-Alexa (**9**), and [G-4]-(coumarin)$_{20}$-Alexa (**10**). FITC = fluorescein isothiocyanate.

Alexa (**9**) and [G-4]-(coumarin)$_{20}$-Alexa (**10**), respectively, each having an average of one Alexa dye at the chain ends, and in the latter case, approximately 20 coumarin units were also attached to the internal dendrimer framework.

To understand the internalization and intracellular trafficking of the unloaded dendritic carrier with internal hydroxy groups, B16 mouse melanoma or HeLa cells were incubated with [G-4]-FITC (**8**) at 37 °C and 4 °C. Subcellular confocal fluorescence microscopy images reveals membrane binding at both temperatures but internalization only occurs at 37 °C (Figure 3a). The cells were then washed to remove excess dendrimer with the formation of endocytic vesicles observed at ca. 2 h and final intracellular targeting to the perinuclear region in about 8 h at 37 °C. Similar results were obtained for the corresponding Alexa647-labeled dendrimer, [G-4]-Alexa (**9**) (see Supporting Information).

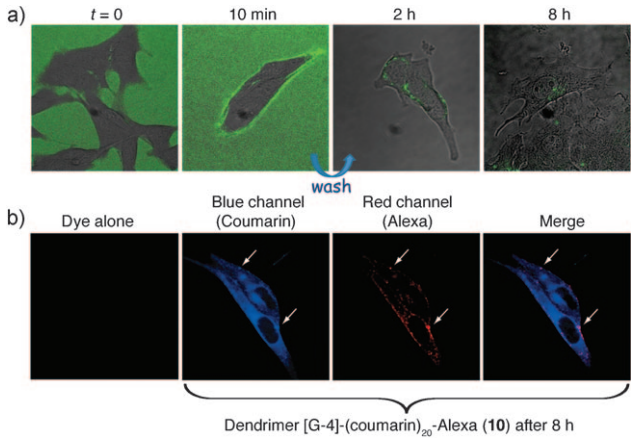


Figure 3. Subcellular confocal images of B-16 cells treated with a) dendrimer [G-4]-FITC (**8**; green) and b) dendrimer [G-4]-(coumarin)$_{20}$-Alexa (**10**; coumarin, blue; Alexa, red) at 8 h.

This behavior is in agreement with previously reported results for the internalization of cationic polyamidoamine (PAMAM) dendrimers, suggesting that after endocytosis the dendrimer is delivered to the lysosome.[18] Having verified the internalization of the hybrid dendritic scaffold, the B16 cells were incubated with the internally functionalized [G-4]-(coumarin)$_{20}$-Alexa dendrimer (**10**) which allows both the dendritic scaffold and the coumarin payload to be tracked at the same time. While the dye alone showed no internalization, indicating that it is not membrane-permeable in its free form, the images of dye-loaded dendrimer show coumarin release inside the cells; a blue fluorescence signal is observed in the cytoplasm and this signal intensity increases over a period of 8 h (Figure 3b). The red signal from Alexa647 dye indicates that the dendritic scaffold is localized only inside endocytic vesicles, probably due to its large size and hydrophilicity and is not able to escape the vesicle membrane. Significantly, co-localization between the blue (payload) and red (scaffold) signals in the vesicles was observed (Figure 3b and Supporting Information), indicating that the coumarin units are released from the dendrimer before escaping the vesicles. Additionally, the payload carrying scaffold, [G-4]-(coumarin)$_{20}$-Alexa dendrimer (**10**), displays a slightly lower internalization rate in comparison to labeled derivative with no functionalization of the internal hydroxy groups, [G-4]-Alexa (**9**). This can be attributed to the more hydrophobic interior due to the presence of the internal coumarin units. These results demonstrate the power of this dual functionalization strategy in providing well-defined delivery systems, which allow for fundamental insights into the intra-cellular behavior of both scaffolds and their payloads.

In summary, we have introduced a novel strategy for the facile synthesis of orthogonally functionalized hybrid dendritic–linear delivery systems, incorporating reactive groups at the chain ends as well as internally, through a combination of "amine–epoxy" and "thiol–yne" click chemistry. Starting from bis-amino-PEG, a fourth-generation dendritic scaffold having chain-end amino groups, a single FITC or Alexa dye, and 20 internal coumarin units could be prepared. Even with this high loading of coumarin units (ca. 25 wt %), confocal microscopy on living cells revealed that upon membrane binding and internalization of the scaffold, intracellular enzymes cleaved the coumarin payload, allowing release into the cytoplasm. As a result, both the dendritic scaffold and the release of its payload can be monitored simultaneously inside living cells.

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