Multivalent Cucurbituril Dendrons for Cell Membrane Engineering with Supramolecular Receptors

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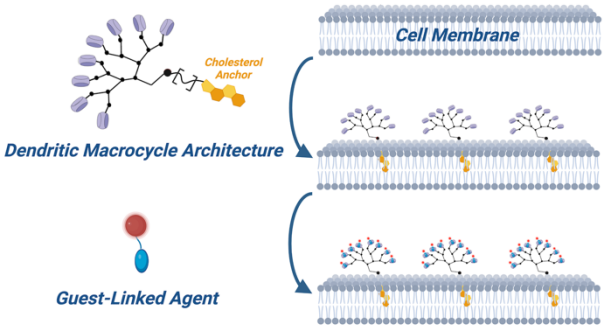
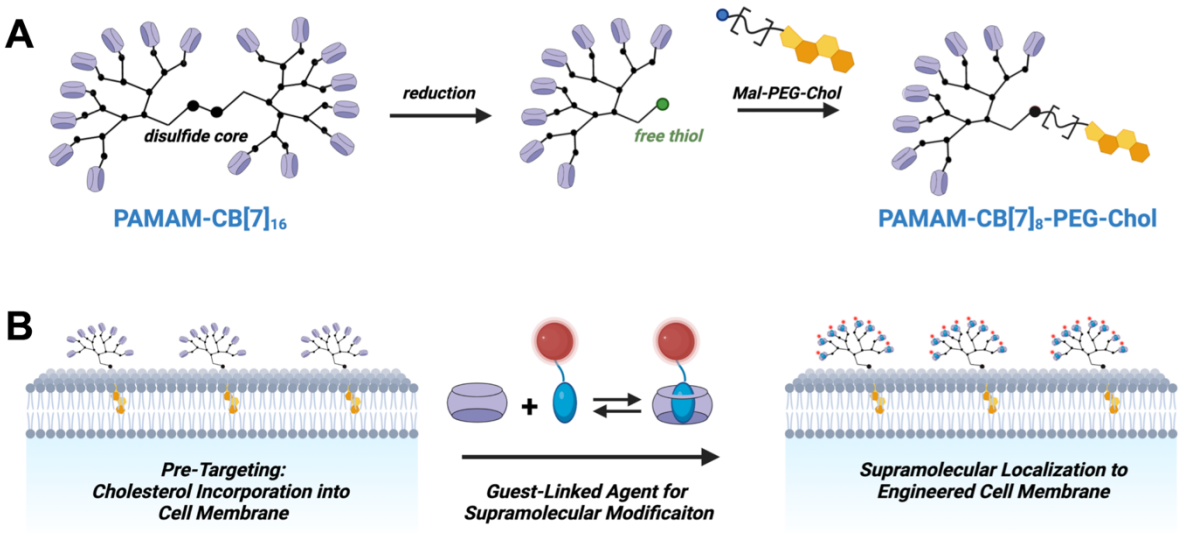
ABSTRACT: The affinity possible from certain supramolecular motifs rival that for some of the best-recognized interactions in biology. Cucurbit[7]uril (CB[7]) macrocycles, for example, are capable of achieving affinities in their binding to certain guests that rival that of biotin–avidin. Supramolecular host–guest recognition between CB[7] and certain guests have been demonstrated to spatially localize guest-linked agents to desired sites *in vivo*, offering opportunities to better exploit this affinity axis for applications in biomedicine. Herein, architectures of CB[7] are prepared from a polyamidoamine (PAMAM) dendrimer scaffold, installing a PEG-linked cholesterol anchor on the opposite end of the dendron to facilitate cell membrane integration. Cells are then modified with this dendritic CB[7] construct *in vitro*, demonstrating the ability to deliver a model guest-linked agent to the cell membrane. This approach to realize synthetic supramolecular "membrane receptors" may be leveraged in the future for *in situ* imaging or modulation of cell-based therapies or to facilitate a synthetic supramolecular recognition axis on the cell membrane.



KEYWORDS: Drug delivery; Supramolecular Chemistry; Dendrimers; Cellular Engineering

The non-covalent recognition realized from directional supramolecular motifs has been explored in recent years for uses in drug delivery, seeking improved drug carriers or new routes to target therapeutics on the basis of affinity.[1,2] Of the many supramolecular motifs, those consisting of host macrocycles that recognize and bind a complimentary guest are among the most well-explored. Host–guest interactions can be designed with affinities (K_{eq}) spanning orders of magnitude, and may furthermore be tunable in response to relevant physiological stimuli.[3] Cyclodextrin is one macrocycle host with a long history of clinical use as a pharmaceutical excipient, solubility enhancer, and drug carrier.[4–7] Though readily synthesized through enzymatic processes from a starch feedstock, cyclodextrins do not typically bind with K_{eq} in excess of 10^5 M^{-1} to any guest.[8] This relatively modest affinity limits the application of cyclodextrins for monovalent recognition and supramolecular complex formulation under dilute conditions.[9] Yet, there are opportunities to be realized from high-affinity host–guest interactions by enabling recognition in complex environments, for instance in mimicking biological recognition motifs like biotin–avidin.[10] Cucurbit[n]uril (CB[n]) macrocycles, prepared from cyclic polymerization of 'n' glycoluril monomers, are one of the few classes of macrocycles capable of accessing the requisite high-affinity binding to achieve this goal.[11–13] One member of this family, CB[7], is a water-soluble variant that forms monovalent complexes with guests that can reach K_{eq} values of at least ~10^{15} M^{-1} in buffer.[14] CB[7] recognition and complex formation *in vivo* has been demonstrated for guests with K_{eq} in excess of 10^{10} M^{-1} and explored for targeting drugs and imaging agents to desired sites in the body.[15–17]

Polyamidoamine (PAMAM) dendrimers are branched macromolecules prepared from a diamine core through divergent dendritic synthesis by repeating amide and amine units.[18] This class of dendrimers is among the most explored for biomedical applications as a result of their excellent biocompatibility compared to other dendrimer families such as poly(propyleneimine), including features



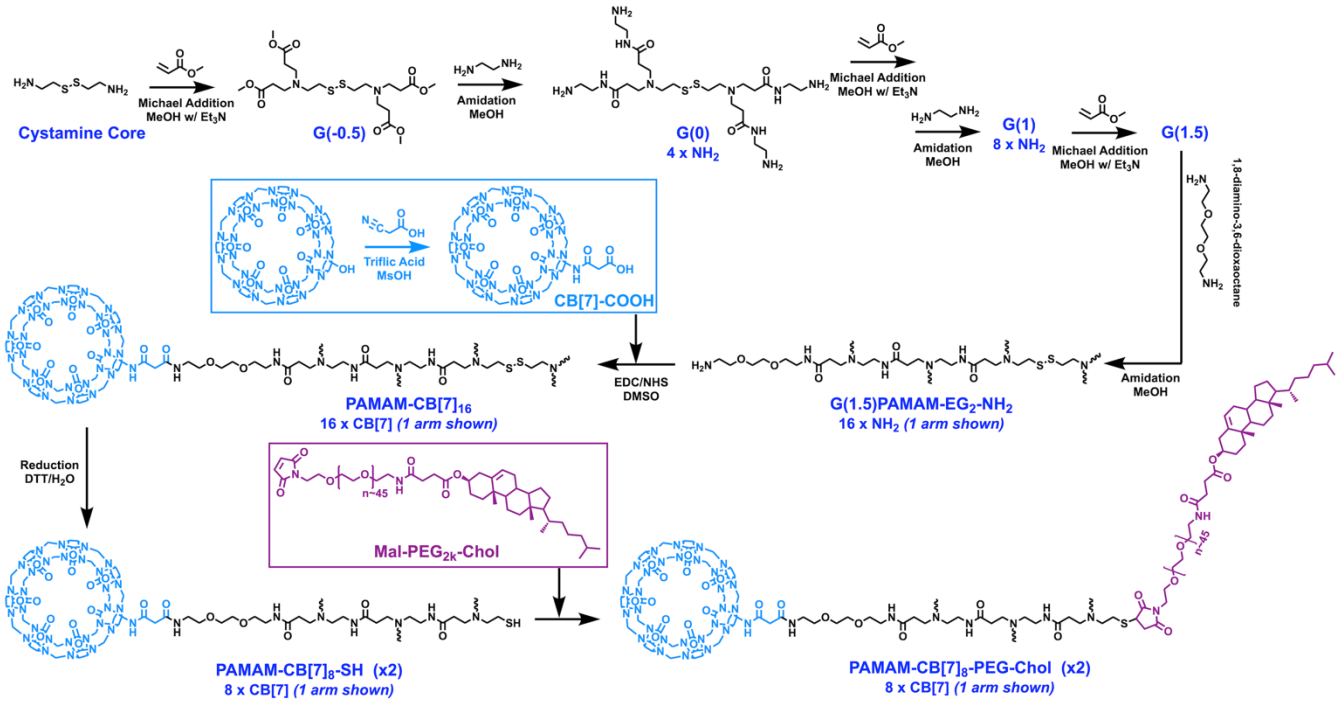
Scheme 1: (**A**) Cartoon schematic illustrating the route to attach a PEG-linked cholesterol anchor to a dendritic CB[7] PAMAM architecture. (**B**) With this approach, the cholesterol anchor is intended to facilitate cell membrane modification with synthetic "membrane receptors" presenting a high density of CB[7]. Then, guest-linked agents can be delivered to the cell membrane on the basis of CB[7]–guest affinity.

such as biodegradability, low immunogenicity, and water solubility.[19,20] PAMAM synthesis alternates between a Michael addition of methyl acrylate to amine-terminated ends to prepare so-called half generations (*i.e.*, G(-0.5), G(0.5), etc.) followed by amidation with ethylenediamine to yield a new amine-terminated surface on the so-called whole generations (*i.e.*, G(0), G(1), etc.).[21] Each cycle of this two-step procedure leads to dendrimer growth, with branching occurring at each half-generation; synthesis can be optimized to control size, shape, molecular weight, and reactivity.[21,22]

Strategies to engineer the cell membrane have been explored to endow prosthetic functionalities, including immune protection, delivery of drugs or other agents, or cell-cell recognition.[23,24] Cell surfaces can be viewed as diverse chemical scaffolds that offer routes for modification by genetic, metabolic, non-covalent, or chemical means. Most of the explored non-genetic approaches have used covalent conjugation, metabolic glycoengineering, and hydrophobic insertion to modify cell membrane chemistry.[25–29] As these techniques do not involve the manipulation of cells at the genetic level, they may be more readily applied to a range of cells from different origins. These routes may furthermore be amenable for inclusion in routine *ex vivo* processing in conjunction with cell-based therapies for transient modification of cell surface chemistry.[23] A generalized approach was therefore explored here to afford supramolecular recognition at the cell membrane through hydrophobic insertion of an asymmetric PAMAM dendron comprising 8 CB[7] motifs opposite a cholesterol anchor (***Scheme 1***).

To realize this supramolecular receptor for cell membrane engineering, G(1.5) PAMAM dendrimers were first synthesized divergently from a cystamine core, yielding a surface terminated with 16 methyl ester groups (***Scheme 2***). Routine synthetic methods were employed,[30] alternating between addition of ethylenediamine and methyl acrylate, with details found in the *Electronic Supporting Information*. The successive growth and purity of dendrimers were verified at each stage using ^{1}H-NMR (***Fig S1-S5***). Upon realizing a G(1.5) PAMAM dendrimer, the final diamine addition instead used 1,8-diamino-3,6-dioxaoctane, effectively facilitating a short ethylene glycol (EG_2) spacer at each of the 16 terminal ends, labeled **G(1.5)PAMAM-EG_2-NH_2** (***Fig 1B, Fig S6***). This addition was designed to reduce the steric limitation for subsequent CB[7] addition. Each step of dendrimer synthesis, including terminal addition of the EG_2 spacer, was effectively quantitative and care was taken to remove unreacted materials to prevent simultaneous growth of lower-order dendrimers.

A longstanding challenge in CB[n] chemistry is accessing macrocycles with functional handles for covalent conjugation, though routes to prepare modified CB[7] macrocycles are now reported.[31–33] Following methods for the direct hydroxylation of CB[7] with ammonium persulfate followed by column chromatography with CHP20P resin,[33,34] **CB[7]-OH** was isolated from CB[7] at ~21% yield. Next, superacid-mediated functionalization with cyanoacetic acid was performed as described,[32] with the pure **CB[7]-COOH** product isolated at ~81% yield (***Scheme 2, Fig 1B***). By carbodiimide-mediated coupling, CB[7]-COOH was conjugated to G(1.5)PAMAM-EG_2-NH_2 *via* an amide linkage to yield **PAMAM-CB[7]$_{16}$** (***Scheme 2,***



Scheme 2: Full synthetic procedure to prepare the PAMAM-CB[7]$_8$-PEG-Chol dendron. PAMAM dendrimers were prepared through divergent synthesis from a cystamine core by alternating Michael addition (w/ methyl acrylate) and amididation (w/ ethylenediamine) reactions. Following preparation of G(1.5), the final amidation used 1,8-diamino-3,6-dioxaoctane to offer a short ethylene glycol spacer. In parallel, CB[7]-COOH was synthesized from CB[7]-OH by superacid-mediated addition of cyanoacetic acid for conjugation to the amine-modified dendrimer periphery. The resulting PAMAM-CB[7]$_{16}$ dendrimer was reduced with DTT and reacted with a commercially sourced Maleimide-PEG$_{2k}$-Cholesterol conjugate *via* thiol–maleimide conjugation to yield the PAMAM-CB[7]$_8$-PEG-Chol dendron.

Fig 1A). The known proton shifts upon 1:1 binding of CB[7] to excess *p*-xylylenediamine guest (probe) in D_2O were used to verify CB[7] content of the dendrimers as well as improve resolution of CB[7] peaks (***Fig 1B***); the ratio of integrated aromatic signal of the threaded probe (δ = 6.46) to a reference dendrimer peak (δ = 2.59) was used to quantify the number of CB[7] motifs per dendrimer (***Fig S7***). The improved resolution upon addition of *p*-xylylenediamine is evident when comparing ^{1}H-NMR spectra with and without this probe (***Fig S8***). Through this method, each dendrimer was calculated to contain an average of 15.91 CB[7] macrocycles, effectively quantitative conjugation relative to the theoretical maximum of 16 amine sites for conjugation on the dendrimer. This method also confirmed CB[7] portals were accessible for guest binding following conjugation. The quantitative CB[7] modification here is noteworthy given prior efforts to modify dendrimers with CB[7] resulted in a maximum of 12 macrocycles modified onto 64 available sites of a related G4 dendrimer prepared *via* alternating epoxy-amine and thiol-yne reactions, with a monofunctional CB[7]-N_3 added to terminal alkynes *via* copper-catalyzed click chemistry.[35] This difference in functionalization efficiency may be due to added steric limitations for this prior dendrimer platform, arising as a result of both the greater density of branching of its chemistry as well as lack of a terminal spacer such as the EG_2 group used here.

Following synthesis of PAMAM-CB[7]$_{16}$, the disulfide in the cystamine core was reduced using DTT to yield two identical dendrons with reactive thiol functional groups. Due to the efficiency and selectivity of maleimide-thiol reactions, maleimide-PEG$_{2000}$-cholesterol was chosen to react to thiols on the reduced dendrons to realize an asymmetric dendron with a membrane-incorporating hydrophobic cholesterol anchor opposite a dendritic CB[7] architecture, termed **PAMAM-CB[7]$_8$-PEG-Chol** (***Scheme 2, Fig S9***)**.** PEG$_{2000}$ afforded a spacer designed to provide sufficient distance between the cell membrane and branched CB[7] dendron to ensure CB[7] accessibility for guest binding after membrane incorporation. A control dendron without the cholesterol anchor was synthesized by similar means from maleimide-PEG$_{2000}$ to yield **PAMAM-CB[7]$_8$-PEG** (***Fig S10***).

Of relevant interest for the functional use of PAMAM-CB[7]$_8$-PEG-Chol dendrons was their ability to embed in the cell membrane through the cholesterol anchor for subsequent host–guest recognition and payload delivery at the cell membrane (***Scheme 1***). NIH/3T3 cells, an adherent fibroblast line isolated from a mouse embryo, were chosen as a model cell to study membrane

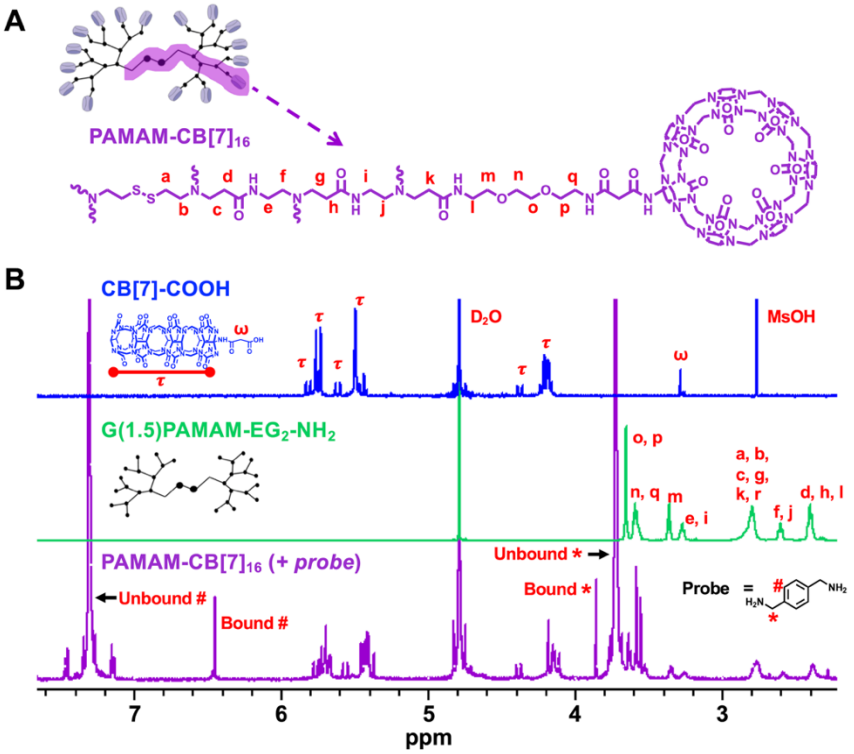


Figure 1: (**A**) Chemical structure of the core and one arm of the PAMAM-CB[7]$_{16}$ dendrimer with relevant proton assignments for the dendrimer portion noted. (**B**) ^{1}H-NMR spectra of CB[7]-COOH (*top, proton assignments noted as Greek letters*), G(1.5)PAMAM-EG$_2$-NH$_2$ (*middle, protons assigned with letters as noted in Part A*), and PAMAM-CB[7]$_{16}$ dendrimer (*bottom, proton assignments of the bound or unbound probe noted as symbols*). All spectra collected in D_2O.

incorporation of PAMAM-CB[7]$_8$-PEG-Chol. A model guest prepared by conjugating ferrocene to a near-infrared fluorescent sulfo-Cy5 dye using a protonating tertiary amine linker (**Fc-N-Cy5**) was selected to visualize capture at the surface of cells and verify CB[7] presentation; this model guest-linked dye was previously reported with a measured K_{eq} for binding to CB[7] of 1.5 × 10^{12} M^{-1}.[15] Different treatment concentrations of PAMAM-CB[7]$_8$-PEG-Chol were explored spanning a range from 10-500 μg/ml dissolved in DMEM, followed after 24 h by addition of 300 μM Fc-N-Cy5 and fluorescence microscopy. A concentration-dependent increase in fluorescence signal was apparent (***Fig S11***), though conventional inverted microscopy did not offer definitive evidence for the spatial localization of fluorescence signal.

Some evidence of solubility limitations for the highest concentration of PAMAM-CB[7]$_8$-PEG-Chol explored in initial experiments pointed to a maximum concentration of 333 μg/ml for future studies. After 3T3 cells were exposed to this agent for 24 h, confocal laser scanning microscopy (CLSM) was next performed with aid of a CellBrite™ Green Cytoplasmic Membrane Dye (***Fig 2A***). As before, CB[7] incorporation was imaged through addition of 300 μM of the guest-linked dye, Fc-N-Cy5. At a concentration of 333 μg/ml PAMAM-CB[7]$_8$-PEG-Chol, the dendrimer has a concentration of CB[7] of 187 μM. Thus, 300 μM of Fc-N-Cy5 ensured an excess; affinity of 1.5 × 10^{12} M^{-1} dictates an equilibrium for this guest–dye conjugate strongly favoring the bound state. Evidence from CLSM showed elevated Cy5 fluorescence signal for cells treated with PAMAM-CB[7]$_8$-PEG-Chol compared with untreated cells stained with the same Fc-N-Cy5 dye. Moreover, strong spatial overlap between the Cy5 and green fluorescent signals supports membrane incorporation of the dendrimer (***Fig 2A, Movie S1***).

Flow cytometry was next used to quantify the increase in Fc-N-Cy5 fluorescence as a function of membrane treatment protocol (***Fig 2B***). Compared to cells that were untreated, fluorescence signal for cells treated for 24 h with PAMAM-CB[7]$_8$-PEG-Chol was increased by ~2 orders of magnitude. Interestingly, cells treated with PAMAM-CB[7]$_8$-PEG lacking the cholesterol anchor demonstrated an increase in fluorescence of ~1 order of magnitude compared to the untreated control. In all cases, cells were stained with Fc-N-Cy5 regardless of membrane treatment. As CB[7] is well-known to bind amino acid side-chains of proteins,[36–38] retention of multivalent CB[7] dendrons on the cell membrane is to be expected. Moreover, the cationic core of PAMAM dendrimers is known to promote some electrostatic

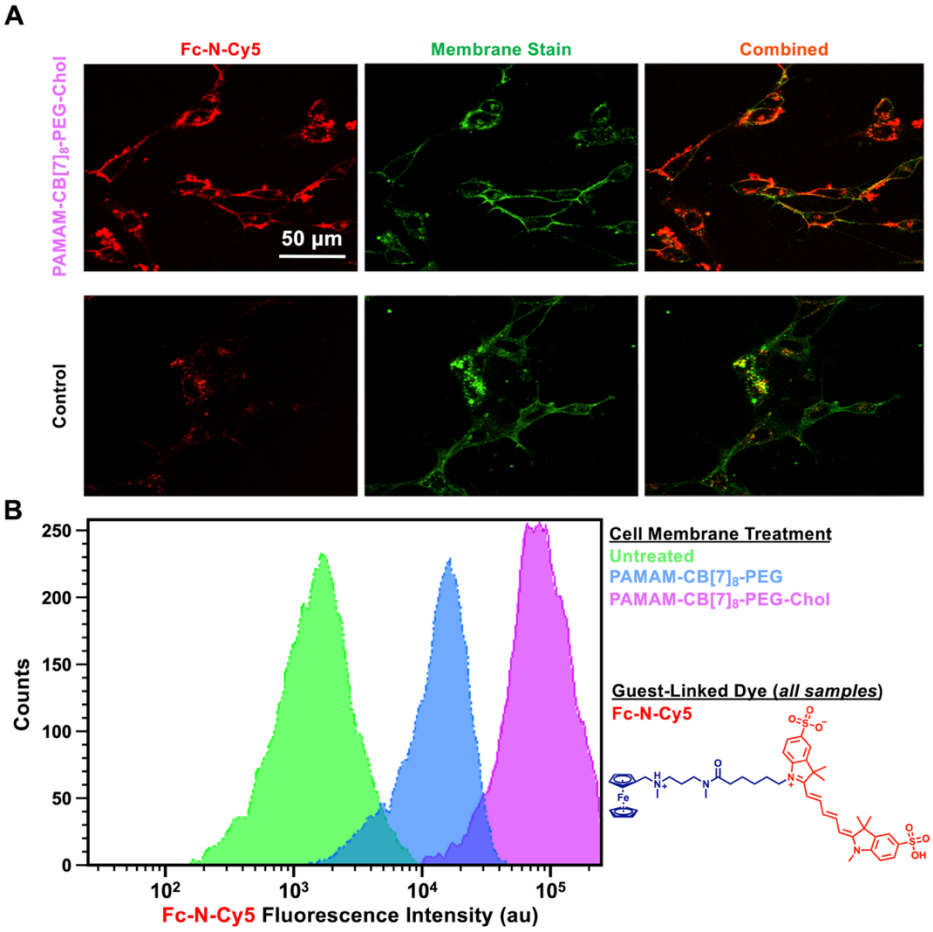


Figure 2: (**A**) Confocal laser scanning microscopy on 3T3 cells treated with PAMAM-CB[7]$_8$-PEG-Chol (*top*) compared to cells untreated by dendrimer. After 24 h, both groups were stained with a guest-linked dye (Fc-N-Cy5) and CellBrite™ Green Cytoplasmic Membrane Dye. Images for the the Cy5 and green channels shown alongside an overlay of both colors. The 50µm scale bar applies to all images. (**B**) Flow cytometry of 3T3 cells treated with PAMAM-CB[7]$_8$-PEG-Chol compared to cells treated with a control of PAMAM-CB[7]$_8$-PEG or untreated by dendrimer. After 24 h of treatment, all groups were stained with a guest-linked dye (Fc-N-Cy5) prior to analysis.

retention on the cell membrane.[39] Yet the presence of the cholesterol anchor is vital to enhancing dendron retention and increased CB[7] presence on the cell membrane. Other lipid anchors, such as DPPE, have been used in a similar capacity to anchor polymers to the cell membrane.[40] However, preliminary designs exploring this lipid as an anchor were not successful at promoting membrane retention on the surface of cells (***Fig S12***). This further speaks to the importance of cholesterol integration into the membrane in anchoring these relatively large dendritic architectures.

The durability of membrane modification with PAMAM-CB[7]$_8$-PEG-Chol, as well as the impact of longer incubation times, was next explored to assess the utility of CB[7] presentation for subsequent therapeutic delivery to membrane-engineered cells. As before, CLSM and flow cytometry were tracked following a period of 1, 2, or 3 d incubation with PAMAM-CB[7]$_8$-PEG-Chol (***Fig 3***). By both CLSM and flow cytometry, a clear reduction in Cy5 signal was evident as incubation time for PAMAM-CB[7]$_8$-PEG-Chol was increased. Between 24 h and 48 h, the average fluorescence intensity by flow cytometry decreased to ~35%. These data support rapid membrane integration of the construct, as the signal was actually reduced upon longer incubation times. Given the rapid doubling time of 3T3 cells (~22 h[41]), some reduction may be expected as signal from the engineered membrane is diluted upon cell division. However, data obtained at 72 h showed a fluorescence signal that was only slightly elevated above cells untreated with dendrimer. Accordingly, while the mechanism for a time-dependent reduction in presentation efficiency is not clear, this finding may be attributable to dilution upon cell division, internalization of the dendron,[42] *in vitro* degradation of amide bonds in the PAMAM core or those linking the CB[7],[43] and/or degradation of the thiol-maleimide bond attaching the dendrimer to the PEG-Cholesterol linker.[44] As the Fc-N-Cy5 guest-dye is not readily taken up by cells, CLSM did not offer insight into the possibility of cell internalization or intracellular processing of the

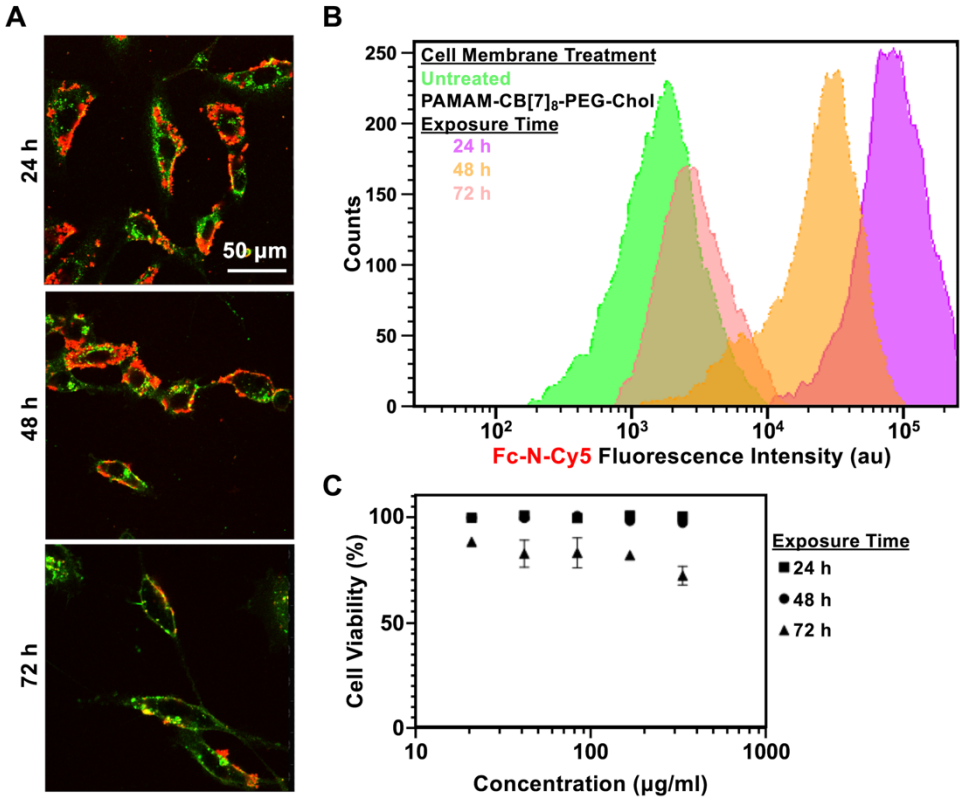


Figure 3: (**A**) Confocal laser scanning microscopy on 3T3 cells treated with PAMAM-CB[7]$_8$-PEG-Chol for 24, 48, or 72 h. In each case, cells were stained with a guest-linked dye (Fc-N-Cy5) and CellBrite™ Green Cytoplasmic Membrane Dye. The overlay of both colors is shown at each time. The 50μm scale bar applies to all images. (**B**) Flow cytometry of 3T3 cells treated with PAMAM-CB[7]$_8$-PEG-Chol for 24, 48, or 72 h compared to an untreated control. All groups were stained with a guest-linked dye (Fc-N-Cy5) prior to analysis. (**C**) Results from an alamarBlue™ cell viability assay for treatment of 3T3 cells with PAMAM-CB[7]$_8$-PEG-Chol for 24, 48, or 72 h at various concentrations, with viability represented by normalizing cell number to an untreated control incubated for the same duration.

PAMAM-CB[7]$_8$-PEG-Chol dendron. Some cytotoxicity was also evident by 72 h of treatment with PAMAM-CB[7]$_8$-PEG-Chol with viability of ~70% relative to untreated controls at this highest treatment concentration (***Fig 3C***). PAMAM dendrimers modified at their periphery to neutralize surface charge, as is the case here, have been reported to have mild cytotoxicity;[42] the observed cytotoxicity on prolonged exposure is thus consistent with prior observations.

This work has explored a concept of engineering cell membranes with multivalent CB[7] dendrons. As supramolecular affinity is increasingly appreciated and explored for *in situ* recognition,[9] efforts to better integrate these as synthetic receptors alongside living biological systems will become more important. In spite of synthetic challenges relative to more commonly used families of macrocycles, CB[7] offers unique attributes in the context of *in vivo* recognition for the localization of drugs or imaging agents.[15–17] As such, routes to endow living systems with this powerful axis of synthetic supramolecular recognition may be important to explore moving forward. Herein, effectively defect-free PAMAM dendrimers were prepared and efficiently modified with CB[7]. Subsequent reduction of the core and attachment of a cholesterol anchor enabled the use of these dendrons for cell membrane modification and facilitated localization of a model guest-linked small molecule payload to the cell membrane. The ability to target active agents to the membrane of engineered cells on the basis of supramolecular affinity could have future applications in conjunction with cell-based therapy, envisioning scenarios where autologous or exogenous cells could be imaged or modulated *in situ* following therapeutic administration. An underexplored facet of the present system is its multivalency, though one could envision the use of this approach in conjunction with corresponding guest-presenting dendritic architectures to facilitate tight and fully synthetic cell–cell interactions. Though the present design lacked durability in its cell-surface presentation, it may yet enable short-term recognition at the cell membrane; applications requiring more prolonged presentation would still be limited. Ultimately, the present approach offers a synthetic route to cell membrane engineering with synthetic "membrane

receptors" of multivalent macrocycles, introducing an axis of CB[7]-based affinity for recognition at the cell membrane.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed synthetic and experimental methods; ^{1}H NMR molecular characterization; supporting data sets for cell studies.(.PDF)

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Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

MJW gratefully acknowledges funding support for this work from the National Institutes of Health (R35GM137987), the National Science Foundation (BMAT, 1944875), and a 3M Non-Tenured Faculty Award (3M Company). Confocal microscopy was carried out in part in the Notre Dame Integrated Imaging Facility, University of Notre Dame using Nikon A1R-MP Laser Scanning Confocal Microscope. We thank Sara Cole for knowledge, expertise, and time in assisting with technical aspects of this work. We also are grateful to Christopher Addonizio and Adam Braegelman for helpful discussions and insights. Scheme 1 and the TOC graphic were created using BioRender.com.

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