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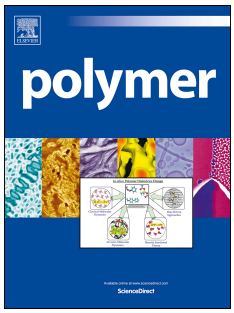

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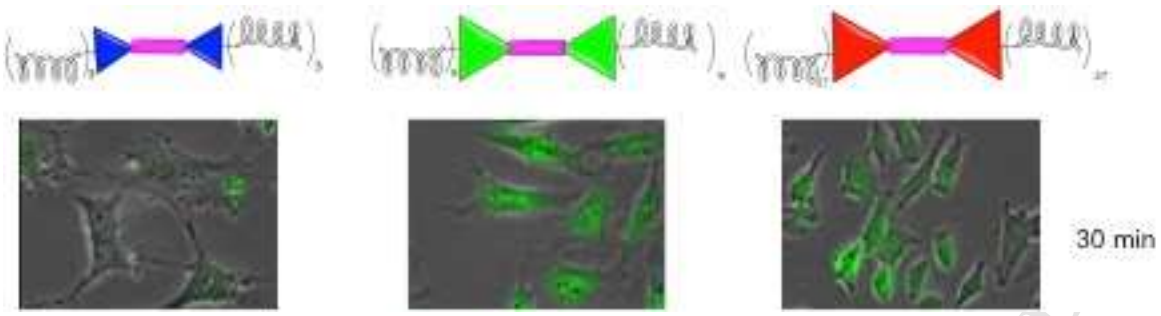

Generation Effect of Newkome Dendrimers on Cellular Uptake

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Abstract: Poly(amide)-based dendrimers can be used as delivery scaffolds in conjunction with the cell-penetrating peptide gH625 derived from the glycoprotein of the *Herpes Simplex* virus type 1. In this contribution, we aim to isolate the optimal dendrimer generation for cellular uptake for Newkome type dendrimers conjugated with gH625. For this study, we synthesized generations zero to three of the Newkome dendrimer-gH625 bioconjugate. Fluorescent microscopy experiments showed that the second and third generations are the most efficient for cellular uptake with the second generation having the synthetic advantage. The optimal second generation can be used as an improved material for a dendrimer based delivery scaffold for peptide therapeutics.

Introduction: Peptide therapeutics have shown promise in various systems but suffer from drawbacks such as protease susceptibility and size limitations common for large therapeutic agents. [1, 2] Therapeutics with molecular weights greater than 500 g/mol show potential for the treatment of a variety of diseases ranging from HIV to cancer [3] but delivery of these drugs from the aqueous extracellular matrix across the amphiphilic bilayer of the cell membrane into cells has proven challenging. [4, 5] Additionally, peptides are vulnerable to proteases and can lead to immune responses in the body. [2] Conjugation

of peptides to polymers can mitigate some of these negatives while allowing for longer circulation times *in vivo* and increased bioavailability. [6, 7]

One promising class of polymer for peptide ligation for biolomedical applications is dendrimers. [8, 9, 10] Dendrimer growth is defined by generations, counting each branching point as a new generation. [9] Increasing the number of termini potentially allows for a higher local concentration of drugs either adsorbed in the dendrimer core or attached to the termini. Synthetic complexity, however, also increases with generation. [11, 12] Often, higher generation dendrimers are less perfect and have a dispersity above 1. In contrast to linear polymers of the same composition, the radius of gyration of a dendrimer grows linearly with generation while the intrinsic viscosity has a maximum value and then decreases when the dendrimer becomes globular. [13] These properties are an advantage when used in biological applications as increasing the size of the scaffold does not greatly effect the viscosity of the intracellular matrix upon delivery. [13] Branched carriers have been shown to be cleared from the kidneys more slowly than their linear counterparts, resulting in longer circulation times giving dendrimers another potential advantage. [7, 14]

Biological systems have been shown to be sensitive to many aspects of polymeric scaffolds. Polymer size, functional density and shape have all been shown to effect cell interactions with polymers. [15 , 16, 17] Thus, optimization of a polymer-peptide conjugate requires careful study of the polymer's activity in a biological application.

Dendrimer generations *in vivo* have been shown to have a marked effect on the behavior of a dendritic drug delivery scaffold. Different generations often show differences in cell uptake and cell toxicity. [18, 19] For example, higher generations of poly(propyleneimine) showed better release of the drug Melphalan, but also a large increase in toxicity. [20] Differences in tumor growth were shown to be negligible between the fourth and fifth generation poly(propyleneimine) dendrimer even as toxicity increased, demonstrating that there is an optimal dendrimer generation for delivery vehicles. [20]

The most widely studied dendrimer for delivery applications is poly(aminoamide) (PAMAM), due to its easy availability and low cytotoxicity when its cationic nature is mitigated. [21, 22] PAMAM dendrimers have been shown to be taken up into cells without the need of cell-penetrating peptides when free amines are present on the termini,

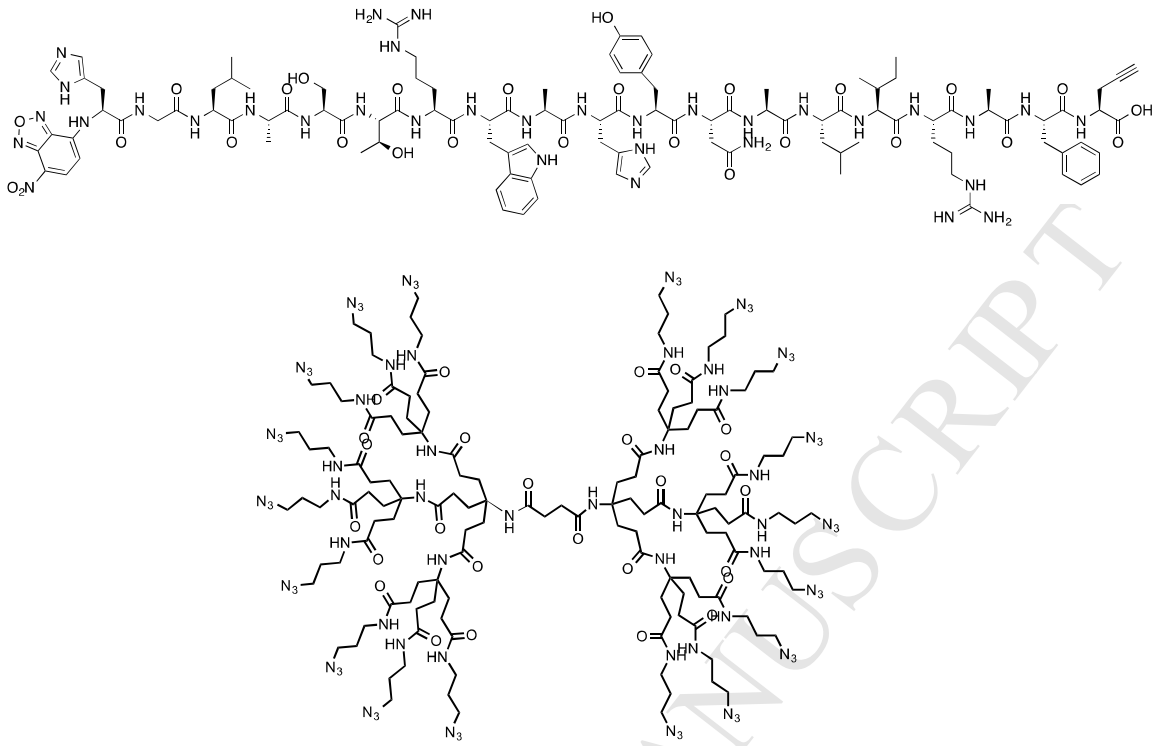


Figure 1. *Previously synthesized second generation azidodendrimer and structure of dye-alkyne modified gH625.*

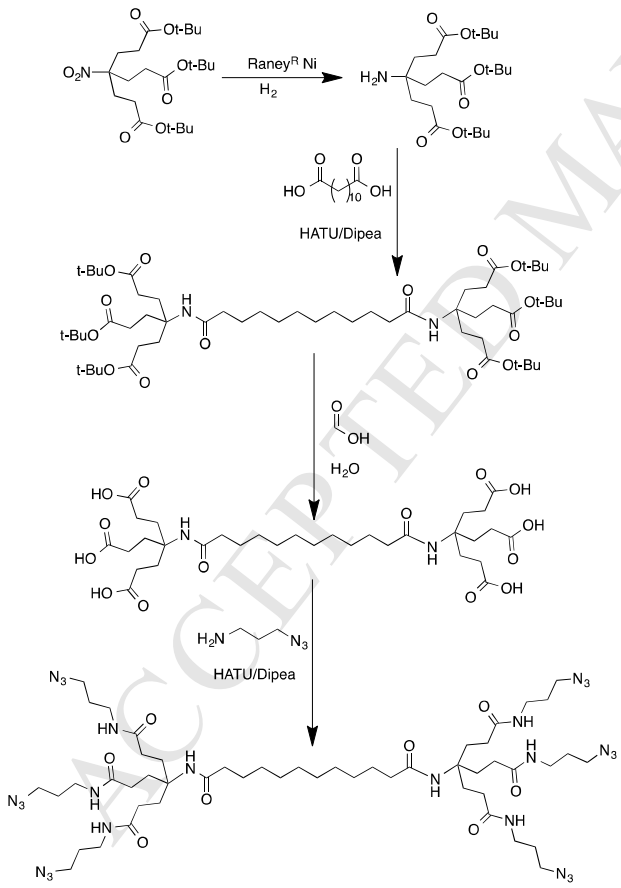
however the dendrimer then shows higher toxicity. [23] Obviously, each of the dendrimer systems used as delivery scaffolds for therapeutics, must be optimized for the desired properties. [24, 25, 26]

The bare dendrimer scaffold is often not enough to deliver cargo into a cell. Building on previous work, our strategy to increase cellular uptake of the scaffold takes advantage of the dendrimer scaffold ligated to the peptide gH625 (Figure 1), derived from a segment of the glycoprotein H from Herpes Simplex virus type 1. [27] This peptide sequence is able to enter the cell and deliver various cargos, a proposed mechanism suggests the amphiphilic nature of gH625's α helical architecture allows the interaction with cellular membranes. [1 , 27, 28]

We have previously demonstrated that compared to free gH625, attachment to the termini of a second generation (G2) Newkome-type dendrimer scaffold greatly increases cellular uptake with low cell toxicity up to 20 μM. [29, 30] At that time, we also performed cell viability assays to determine the optimal concentration of peptidodendrimer based on

the concentration of peptide using UV-vis analysis. This concentration was used in our study to allow for comparison between these findings and previously reported studies.

The uptake was previously measured using both fluorescence microscopy and flow cytometry and it was shown in both cases that the peptidodendrimer had an advantage over free gH625. [29, 30] The use of the G2 dendrimer was wholly arbitrary in our prior research; no comparison was made between various generations of dendrimer. The initial results suggested a potentially new peptide scaffold based on Newkome-type dendrimers functionalized with gH625 as a cell penetrating peptide to deliver payload into HeLa and Vero cells. As a first step towards the optimization of our cell penetrating scaffold, this contribution investigates the generation dependence of our gH625 functionalized Newkome-type delivery scaffold to determine which generation is most suited for further study. We show that there is an ideal size for our scaffold based on cellular uptake, ease of synthesis and maximization of cargo.



Vfkhp h#41# *V| qwkhwlf#vfkhp h#ir u#wkh#iluvw#j hqhudwlr q#ol}lgr ghqgulp hu1*

Results

Our dendrimers of choice are poly(amide)-based with a 1→3 branching unit structurally derived from dendrimers first reported by Newkome, Scheme 1. [31, 32] All dendrimers are synthesized from the commercially available bifunctional dendrons di-t-butyl-4-[2-(t-butoxycarbonyl) ethyl]-4-aminoheptanedicarboxylate (aminotriester) and 4-(2-carboxyethyl)-4-nitroheptanedioic acid (nitrotriacid). The dendrons are coupled using

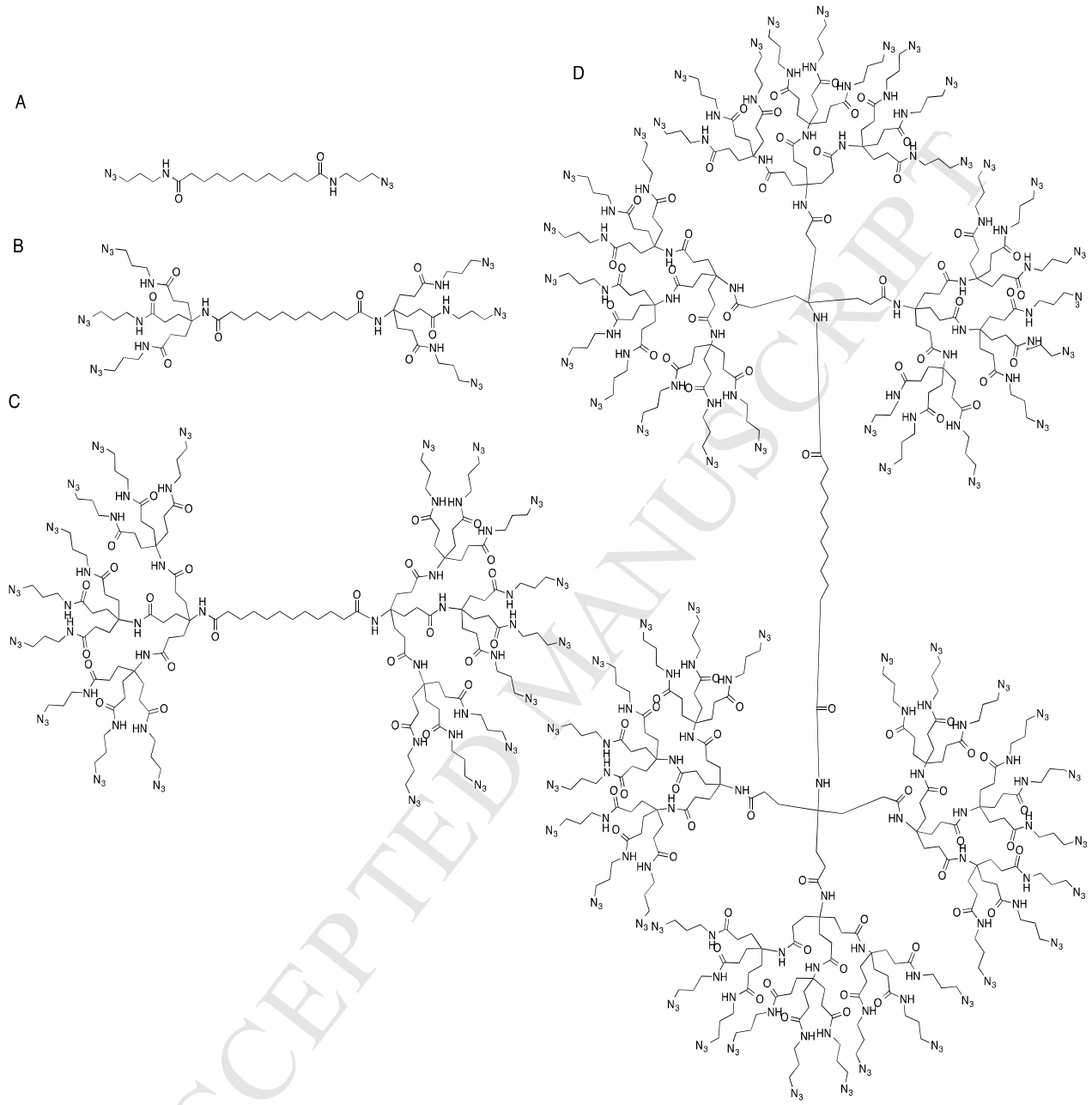


Figure 2. *Structure of the Newkome type dendrimers: A) zeroth, B) first, C) second, and D) third generation azidodendrimers.*

carbodiimide/DIPEA peptide-coupling schemes. The nitro group is reduced subsequently to an amine in order to yield a reactive terminus. The tert-butyl esters are hydrolyzed yielding multiple reactive termini. This strategy can be repeated multiple times to yield the dendrimer of the desired generation (Figure 1). A 3-azidopropylamine linker can be coupled to each terminus of the dendrimer of interest giving a handle to attach alkyne-

functionalized peptides using copper catalyzed 1,3-dipolar cycloaddition. [33, 34, 35] Alternatively, a dendrimer can be directly functionalized using peptide bond coupling strategies. All dendrons are synthesized using iterations of the coupling and deprotecting reactions and detailed syntheses and characterizations can be found in the supporting information. The dendrimers are characterized by ^{1}H and ^{13}C NMR spectroscopies and mass spectrometry.

To visualize the dendrimer in cell culture, a fluorescent tag with a butynyl handle was synthesized in a single step by reacting 4-chloro-7-nitrobenzofuran with 1-amino-3-butyne. The dendrimers were functionalized quantitatively with the fluorescent tag using microwave-assisted copper catalyzed 1,3-dipolar cycloaddition. The gH625 peptide, was synthesized and fluorescently labeled using standard Fmoc solid phase synthesis. An alkynyl handle was added to the C terminus to allow for copper catalyzed 1,3-dipolar cycloaddition. The dendrimers were functionalized with gH625 using again microwave assisted copper catalyzed 1,3-dipolar cycloaddition. The conversions ranged from 72-100% depending on dendrimer generation as calculated by MALDI (Figure 3).

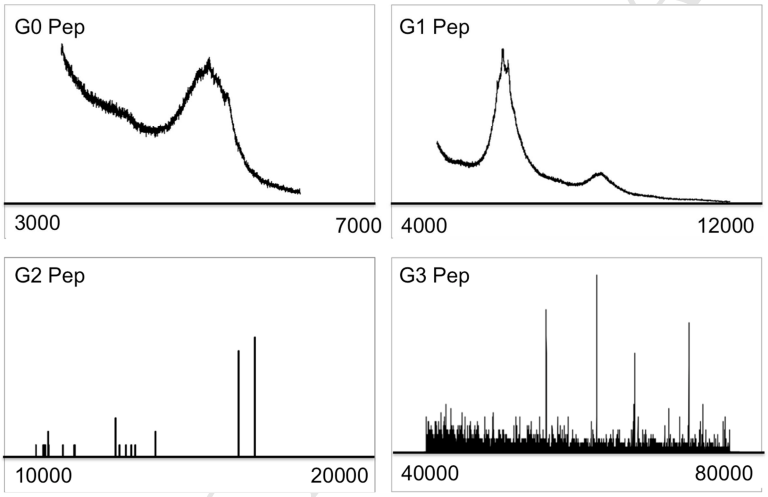


Figure 3. *MALDI of G0-G3 Peptidodendrimers Top left) G0 dendrimer with two peptides: 100% conversion; top right) G1 dendrimer functionalized with six and five peptides: 83.3-100% conversion; bottom left) G2 dendrimer with 18 and 17 peptides: 94.4-100% conversion; bottom right) G3 dendrimer with 54, 48, 44 and 39 peptides: 72.2-100% conversion.*

With the functionalized dendrimers of generations 0-3 in hand, we targeted the effect of generation size on cellular uptake using HeLa cells. Dynamic light scattering (DLS) was run to probe the solubility of the dendrimer dye conjugates at the targeted

concentration to insure that there was not aggregation at experimentally relevant time scales. The larger generations (G2-G3) showed no aggregation in 1% DMSO in buffer and thus 20 μM solution was used as in previous studies of the dendrimer dye conjugate. DLS had previously been run on the G2 peptitodendrimer and thus solubility was not retested. The cellular uptake of each generation of dye-dendrimer conjugate and peptidodendrimer was explored by incubating a solution of the peptidodendrimer of interest in cell medium and HeLa cells for 30 minutes, one hour, two hours, and four hours with a 20 μM solution of each generation of dendrimer relative to the dye. This was calculated by using the extinction coefficient of the 7-nitrobenzofurazan (NBD) dye to determine the concentration of the solution and the desired amount of each dendrimer dye conjugate and peptidodendrimer. The sample was then freeze dried and a stock solution for each cell study was made in 1% DMSO in Dulbecco's modified Eagle's medium.

After incubation, excess peptidodendrimer was washed away and the cells were imaged using fluorescent microscopy (60× PlanFluor objective, NA 0.3, Eclipse TE 2000-U; Nikon) and the fluorescent uptake was calculated (Figures 4 and 5).

The zero generation (G0) dendrimer-dye conjugate had full conversion with two dye molecules per scaffold as calculated by ^{1}H NMR. The G0 peptidodendrimer was fully functionalized with two peptides per scaffold as calculated by MALDI (Figure 3). The G0 dendrimer with dye was taken up more readily by the cells than the G0 peptidodendrimer likely due to the low molecular weight of the compound. The sum of all fluorescent uptake for the zero generation dendrimer-dye conjugate was 996.2 +/- 92.1 rfus (relative fluorescence units) compared to 348.4 +/- 39.0 rfus for the peptidodendrimer (Figure 6). The G0 dendrimer-dye conjugate was able to cross the cellular membrane easily due to the small size of the scaffold. The result shows that the G0 peptidodendrimer is not more readily taken up by cells and is therefore not a viable scaffold for efficient cellular uptake.

The first generation (G1) dendrimer-dye conjugate yielded complete conversion with six dye molecules per scaffold as calculated by ^{1}H NMR. The G1 peptidodendrimer was functionalized with four to six peptides per scaffold as calculated by MALDI. The G1 dendrimer-dye conjugate is taken up less readily by the cells than the G0 dendrimer-dye conjugate. The G1 peptidodendrimer, however, is taken up by the cells more readily than

the control dendrimer but also appears to deposit on the cell membrane leading to cell death (Figure 4). The uptake of the G1 dendrimer also decreases from 194.6 rfus at one hour to 134.1 rfus at two hours as the cells begin to contract. The sum of all fluorescent uptake for the G1 dendrimer-dye conjugate was 125.7 +/- 15.6 rfus compared to 541.8 +/- 71.1 rfus for the peptidodendrimer (Figure 6). The aggregation of the G1 peptidodendrimer, despite the improved uptake, is therefore not a viable scaffold for cellular uptake.

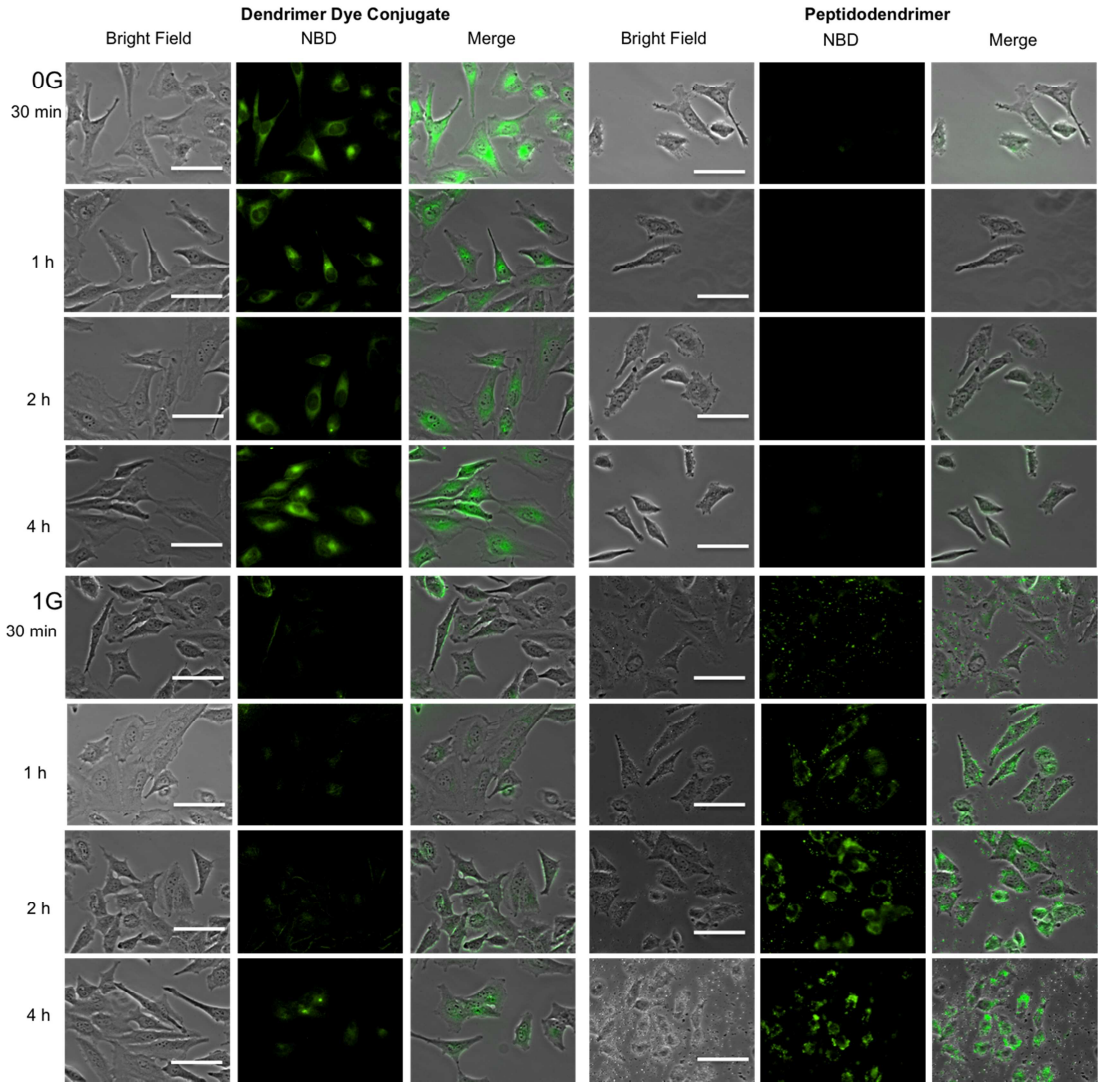


Figure 4. *Microscopy images of cells at 30 min, 1 h, 2 h, and 4 h of incubation with the G0 or G1 dendrimer-dye and peptidodendrimer conjugates. Scale bars represent 50 μM.*

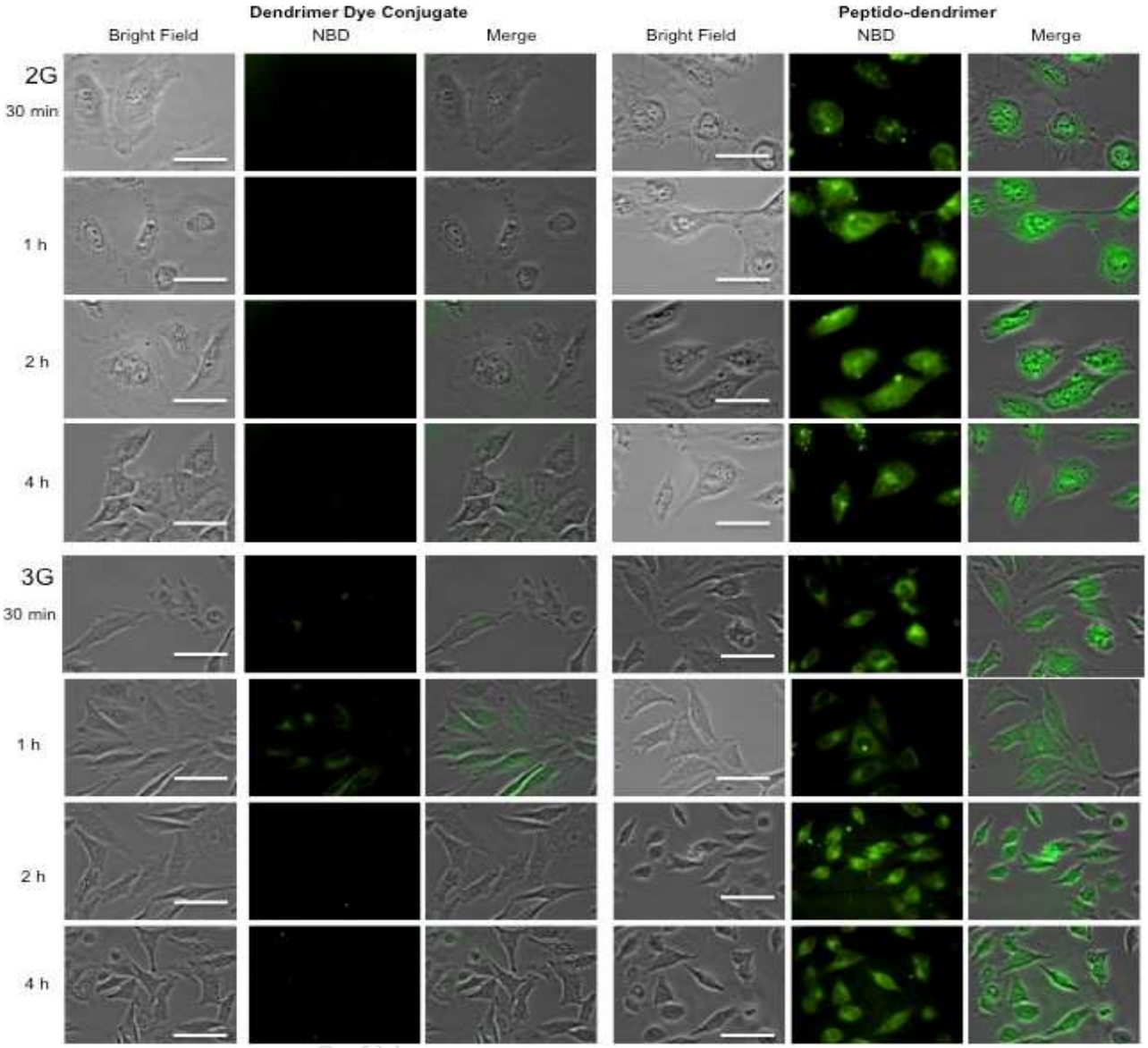


Figure 5. *Microscopy images of cells at 30 min, 1 h, 2 h, and 4 h of incubation with the second or third generation dendrimer-dye and peptidodendrimer conjugates. Scale bars represent 50 μM.*

The G2 dendrimer-dye conjugate was functionalized with eighteen dye molecules per scaffold as calculated by ^{1}H NMR spectroscopy. The G2 peptidodendrimer was functionalized with an seventeen to eighteen peptides per scaffold. The G2 dendrimer-dye conjugate shows similar uptake to the G1. The peptidodendrimer, however, shows significantly higher uptake than the G0 or G1 dendrimers. The uptake is shown to continue to increase as the kinetic study progresses through the four hour time point (Figure 5). The fluorescence of the G2 peptidodendrimer appears diffuse through the

cytoplasm indicating that it is a viable delivery scaffold. The sum of all fluorescent uptake for the G2 dendrimer-dye conjugate was 579.4 +/- 80.1 rfus compared to 2053.0 +/- 340.0 rfus for the peptidodendrimer (Figure 6). These results show that the G2 peptidodendrimer is a viable scaffold for efficient cellular uptake.

The third generation (G3) dendrimer-dye conjugate was functionalized with fifty-four dye molecules per scaffold as calculated by ^{1}H NMR spectroscopy. The G3 peptidodendrimer was functionalized with thirty nine to fifty three peptides per scaffold as calculated by MALDI. The G3 dendrimer-dye conjugate is not readily taken up by the cells. The G3 peptidodendrimer is taken up at a slower rate than the G2 dendrimer, (Figure 5). The fluorescence of the G3 peptidodendrimer appears diffuse through the cytoplasm. The sum of all fluorescent uptake for the G3 dendrimer-dye conjugate was

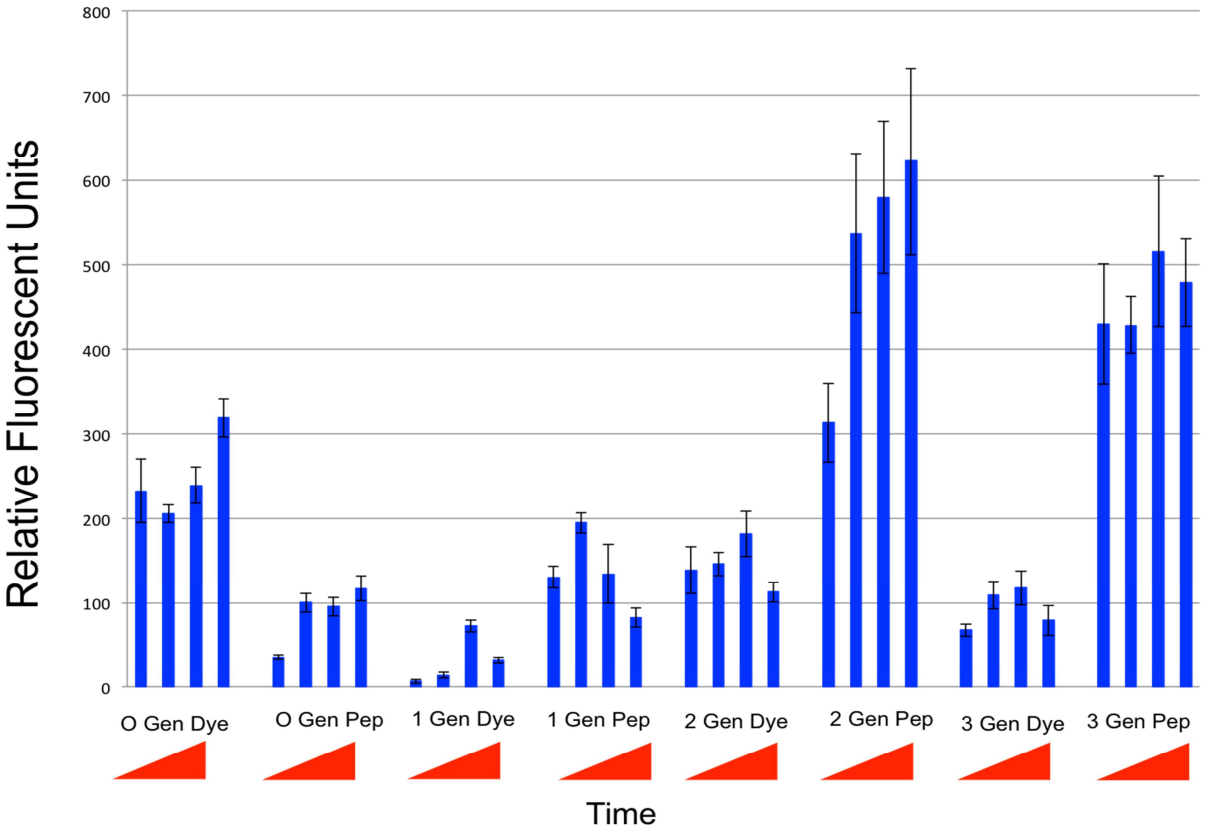


Figure 6. *Raw fluorescent uptake dendrimer-dye conjugates and peptidodendrimer of all dendrimer generations over three biological and nine technical replicates at time points 30 min, 1 h, 2 h, and 4 h.*

373.5 +/- 61.4 rfus compared to 1855.3 +/- 245.0 rfus for the peptidodendrimer (Figure 6). These results demonstrate that the G3 peptidodendrimer is also a viable scaffold for efficient cellular uptake.

The raw relative fluorescence of each dendrimer-dye conjugate and peptidodendrimer conjugate was measured over nine technical replicates, (Figure 6). The trend that emerges with the raw cellular uptake date shows the previously studied G2 peptidodendrimer has the highest uptake overall of peptide. The cell uptake results suggest that the optimal generation for cell delivery is either the G2 or G3 generation dendrimer due to the high uptake into cells. The different generations however have an exponential difference in the number of peptides per scaffold thus the result indicates the number of peptides taken up and not the number of dendrimers.

The maximum number of peptides on each dendrimer scaffold can normalize the raw analysis, which allows to calculate the relative number of dendrimers taken up in each cellular assay (Figure 7). In this analysis, the G0 peptidodendrimer appears to outperform every other generation of dendrimer. This normalization, however does not take into account the difference in dendrimer loading since it is normalized to peptide. There is a nine times higher concentration of G0 dendrimer in solution compared to the G2 dendrimer. When the data are again normalized to the loading of G2 dendrimers, we return to the original relative uptake and the G2 dendrimer again outperforms each generation of peptidodendrimer conjugate. This suggests that the G2 dendrimer is the most efficient for cellular uptake and that there is

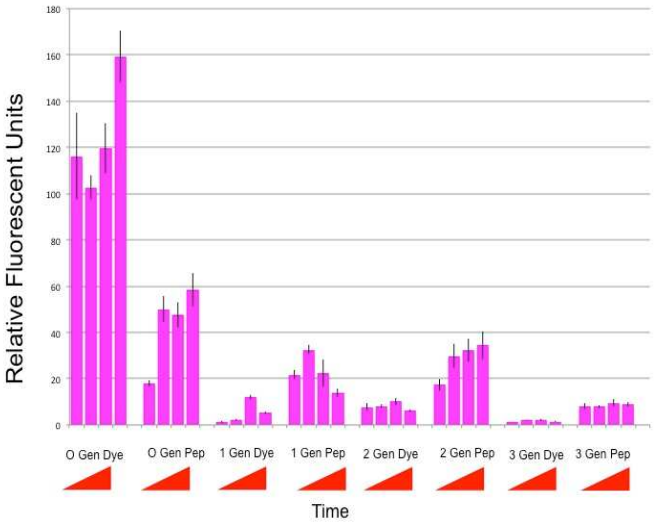


Figure 5. *Fluorescent uptake of dendrimer-dye conjugates and peptidodendrimer for all dendrimer generations over three biological and nine technical replicates at time points 30 min, 1 h, 2 h, and 4 h normalized by number of peptides per scaffold.*

not a significant advantage to the third generation dendrimer.

The difference in the analysis is dependent on what is considered the desirable cargo within the study. If the dendrimer would be considered the active agent, then further studies might be warranted at equal molar concentration of G0 and G2 dendrimer. However, in our system, the peptide itself is the biologically active agent and a cell penetrating peptide. For this reason, the G2 dendrimer still holds an advantage over the G0 dendrimer. With the confirmation that the G2 peptidodentimer is the optimal generation, the cell viability experiments previously run with the G2 dendrimer were not repeated. [29, 30] *The flow cytommetry studies reported in past works have shown the dendrimer peptidodendrimer is able to fully cross the cellular membrane and not simply deposit on the cell as is the free peptide.*

The G2 dendrimer is significantly easier to synthesize and isolate than the third generation. Additionally, the second generation has a slightly higher raw cell uptake (albeit within the error range), thus making it the optimal generation Newkome-type dendrimer for cellular uptake. Taking into account both ease of synthesis and biological activity, the second generation outperforms the third across all criteria.

Conclusions

In this study, the cellular uptake of Newkome-type dendrimers as a function of dendrimer generation was probed. All generations except for the G0 showed improved uptake when conjugated to the cell-penetrating peptide gH625. The G1 peptidodendrimer was able to enter the cells but also deposited on the cell membrane causing cell death. The G2 and G3 peptidodendrimers were found to have significantly improved cellular uptake over their dendrimer-dye controls. This optimization of the peptidodendrimer allows further research into cellular uptake using only the G2 dendrimer with the addition of cargo to be delivered into the cells.

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- Four generations of dendrimer peptide conjugates were synthesized.
- Each peptidodendrimer is able to enter HeLa cells via a combination of active and passive pathways.
- The second generation (G2) peptidodendrimer is the optimal generation for intracellular delivery.