Bioorthogonal Swarming: In Situ Generation of Dendrimers

Bioorthogonal Swarming: *in situ* Generation of Dendrimers

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Supporting Information Placeholder

ABSTRACT: With the aid of bioorthogonal chemistry, we demonstrate the fabrication of synthetic dendrimers *in situ* around living cells. Using tetrazine–dienophile and aminooxyl/hydrazide–aldehyde chemistries, the density of functional groups on the dendrimers exponentially amplified intensities of fluorescent markers in antibody-targeted live cell imaging. This novel "swarming" approach highlights the power of biorthogonal chemistry and provides a route to non-natural chemical structures on cells, paving the way for the generation of various artificial cellular nanostructures and scaffolds.

Dendrimers, with their well-defined structures, high density of functional groups, and a variety of internal binding elements, are ideal carriers for a variety of chemical cargos be they small molecular therapeutics,[1–4] biomacromolecules (DNA, RNA and proteins),[5–9] fluorescent reporters,[10–13] or radioactive isotopes.[14] As such, dendrimers are used in a variety of biological and medical scenarios and are often synthesized and modified with targeting ligands, before biological application.[15–19] Polyamidoamines (PAMAM) are one of the most widely investigated dendrimers and have been explored for imaging,[20,21] gene transfection[22,23] and protein/drug delivery to name but a few,[20, 24] offering precise control over their size, shape and the placement of functional groups (typically through distal primary amine functionalisation).[25,26] Peptide-based dendrimers have also found uses in various biological applications ranging from multiple antigenic peptides (MAPs)[27,28] to artificial capsids[29] and imaging probes.[30] Progress has already been made in fabricating linear polymers (by radical polymerizations) on the cell surface of yeast[31] and even within mammalian cells;[32] however, currently, these polymerization reactions are limited by the choice of monomers and lack of precise structural control.

Here we explore, for the first time, the *in situ* generation of dendrimers within/on biological systems (live cells) – an approach that requires highly bioorthogonal, biocompatible water-based chemistries.[33,34] This *in situ* dendrimer formation offers the possibility of enhanced assay detection technologies, entrapping or "straightjacketing" cells, or the means of joining multiple ligands or cells together. In addition, this approach offers new biological applications of dendrimers, such as *in situ* synthesis of artificial organelles[35–37] and biocatalysis.[38–42]

A number of bioorthogonal reactive partners such as aminooxyl–aldehyde,[43–46] boronic acid–diol,[47–49] tetrazine–dienophile,[50–53] and azide–cycloalkyne,[54] have been used in applications from cellular labelling to material synthesis. The absence of bioorthogonal reactive centers on the surface of cells means that selective attachment with compatible reactive handles, for specific functional molecule conjugation in complex biological environments, is possible (e.g. the classic azido sugar incorporation onto cells[55]).[33] However, the *in situ* generation of dendrimers utilizing these powerful chemical tools has not been reported. Here, multibranched, bifunctional building blocks were synthesized, with bioorthogonal conjugations achieved using tetrazine–dienophile and aminooxyl/hydrazide–aldehyde chemistries, with dendrimer generation validated first in solution and ultimately on cells via antibody targeting (Figure 1). The resulting dendrimers provided multiple reactive centers for bioorthogonal conjugations for amplification of fluorescence signals by means of so-called bioorthogonal "swarming", *i.e.*, rapid exponential amplification of reactive motifs by bioorthogonal conjugation of building blocks to a targeted reactive core.

The three multibranched building blocks **1**–**3**, bearing bioorthogonally reactive centers (aldehyde–NHS ester **1**, norbornene–aminooxyl **2**, and tetrazine–aldehyde **3**), were synthesized using both solid-phase[56] and solution-based chemistries (Figure 1, Schemes S1–S3). The building blocks, by design, contained high densities of amides and poly(ethylene glycol) groups to promote water solubility,

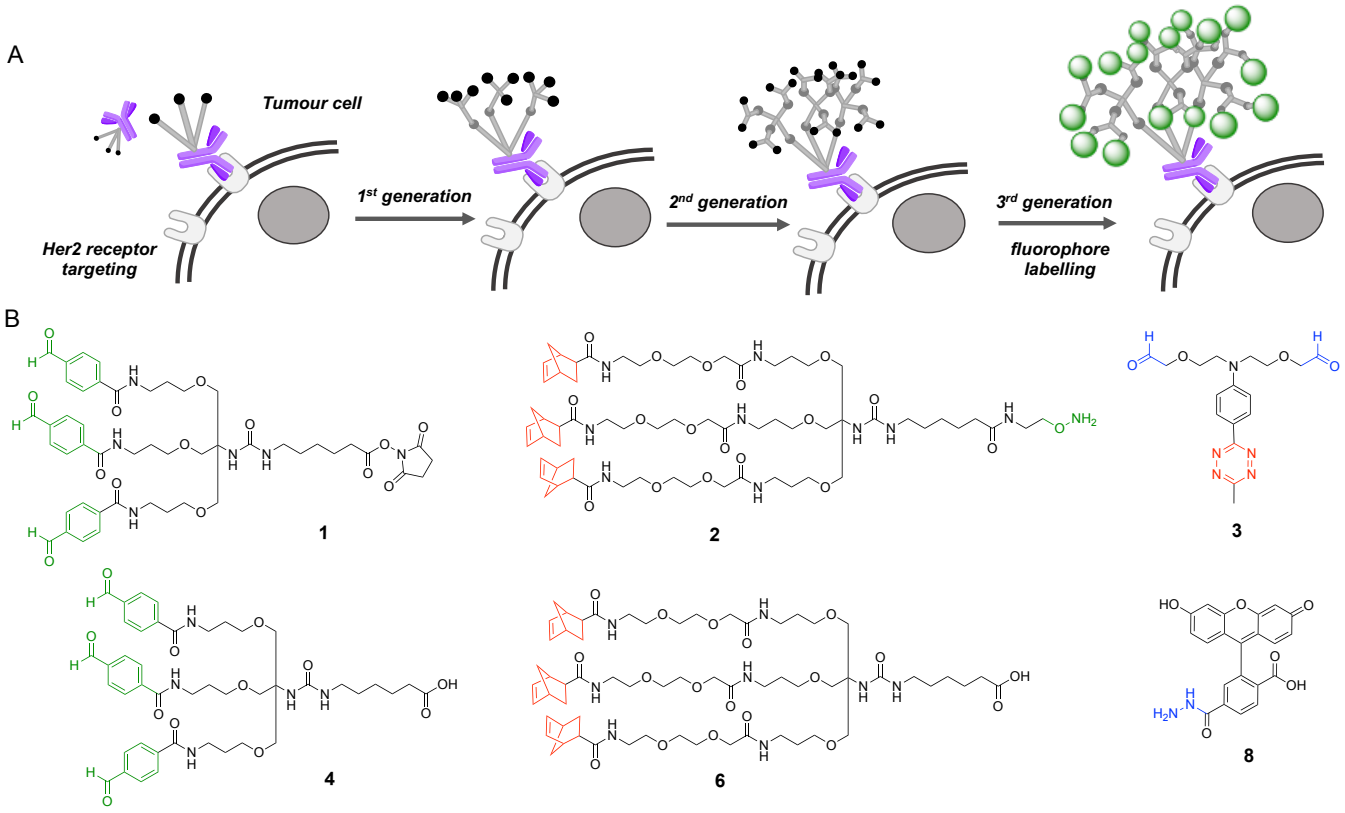


Figure 1. The concept of biorthogonal swarming and the building blocks used in this study. (A) Dendrimer generation by biocompatible, bioorthogonal "swarming" around a HER2 targeting antibody on live cells. (B) The designed, multifunctional building blocks used for dendrimer synthesis and fluorescent labelling.

with spacers between functional moieties minimizing steric congestion.[57,58] The branched building block **1** was designed as the first component of the dendrimer, with the NHS ester allowing conjugation onto primary amines (e.g. on antibodies giving **Her-1**, Figure S1), while the three aldehydes provide amplification sites for aminooxyl or hydrazide ligation (Figure 1). The second building block **2** was constructed with one aminooxyl motif and three norbornenes to enable amplification from three aldehydes to nine norbornenes (**Her-2**, Figure S1). Building block **3** has a single tetrazine and two aldehydes, allowing conjugation with the norbornenes and amplification of the reactive centers to give a total of 18 aldehyde groups (per dendrimer) on the antibody (**Her-3**, Figure S2). Labelling of the final dendrimer was achieved with fluorescein hydrazide **8**[59] (giving the final antibody-dendrimer **Her-4**, Figure S3). The inverse electron-demand Diels–Alder (IEDDA) reaction between a tetrazine and a dienophile, and aminooxyl–aldehyde imine formation, are orthogonal to each other, allowing dendrimer generation.

The chemistries between the individual building blocks were first evaluated/validated by HPLC and ^{1}H NMR. Tris-aldehyde **4** (without the active ester) was used as a model for **1** to investigate imine formation under biologically relevant conditions. When aminooxyl **2** was incubated with tris-aldehyde **4**, full conversion of **4** and the generation of norbornene **5** as the only product was observed within 4 h (Figures 2 and S4). The IEDDA chemistry was evaluated with model norbornene **6** (without the aminooxyl) and tetrazine **3** with full conversion to bis-aldehyde **7** observed by HLPC after 30 min (Figure 2) with ^{1}H NMR characterization showing the efficient generation of **7** (Figure S5). Thus, the oxyimine formation and the IEDDA chemistries were rapidly completed with high product purities and yields (note, the full conversion of the reactions requires completion of three individual reactions).

To investigate full dendrimer formation and fluorescence signal amplification, a sulphonated Cy5[60] fluorophore (bearing a short PEG spacer) was conjugated onto the tri-branched aldehyde **1** via the NHS ester to give **9** (Scheme S4), which was then used to build the fluorescently labelled dendrimer **10** (Scheme 1), using the aminooxyl **2** and tetrazine **3** building blocks and fluorescein hydrazide **8**, consecutively, with the reactions monitored by ^{1}H NMR and MS (Figures S6 and S7). The constructed "first-generation" Cy5-dendrimer **9** was also treated with fluorescein hydrazide **8** (giving **S13**, Figure S8) and both of the labelled dendrimers were analyzed by UV-Vis spectroscopy. The number of fluorescein molecules on dendrimers **S13** (first-generation, 3 aldehydes) and **10**

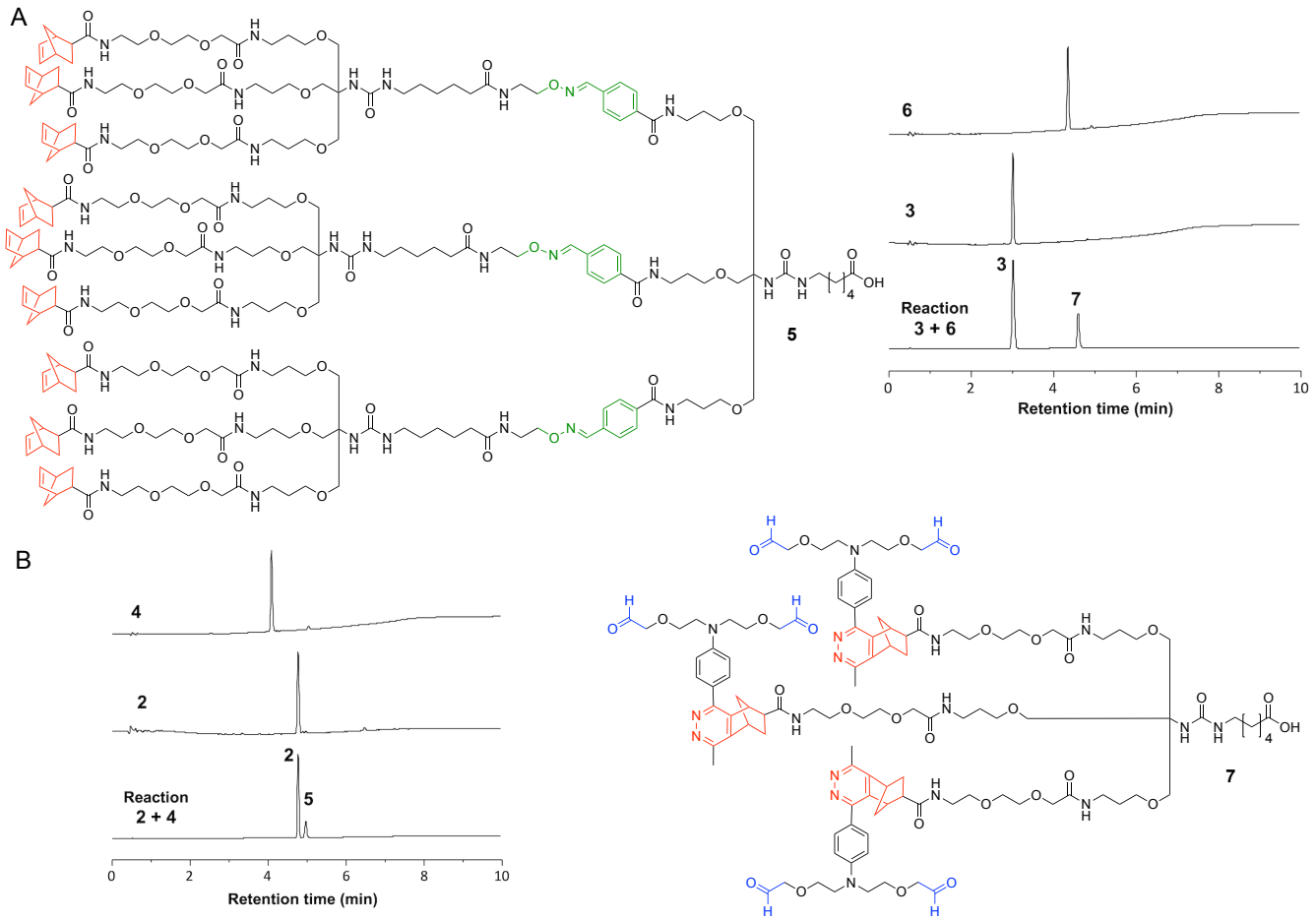


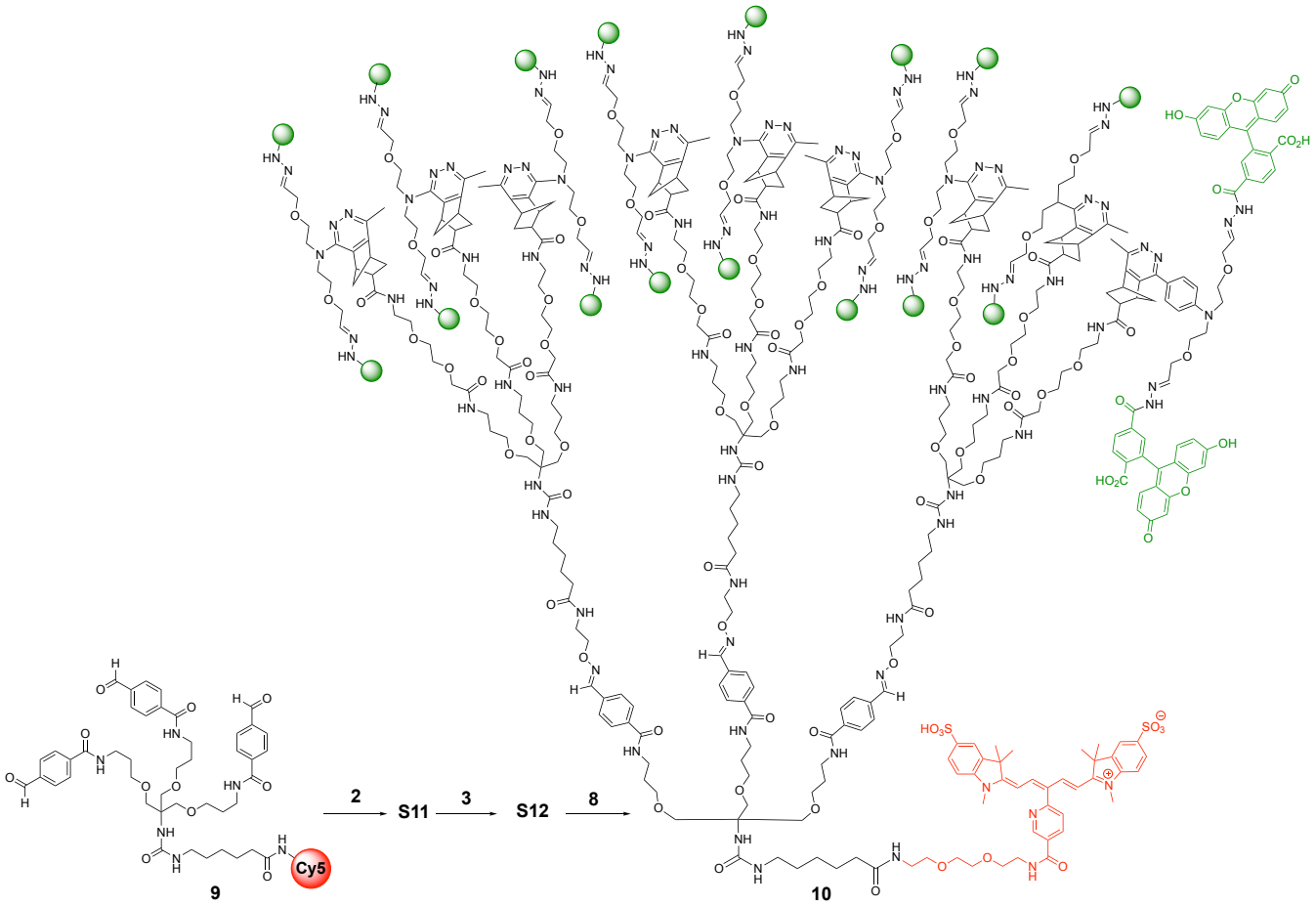
Figure 2. Evaluation of the aminooxyl–aldehyde imine formation and tetrazine–dienophile IEDDA reactions. (A) Model tris-aldehyde **4** (1 mM) was reacted with aminooxyl **2** (15 mM, 5 equiv. per aldehyde) in MeCN/PBS (1:1, v/v) at 37 °C for 4 h to give **5**, with the conversion monitored by HPLC (ELSD detection). (B) Model norbornene **6** (1 mM) was reacted with tetrazine **3** (10 mM, 3.3 equiv. per norbornene) in MeCN/PBS (1:1, *v/v*) for 30 min to give bis-aldehyde **7**, with the conversion monitored by HPLC (ELSD detection).

(third-generation, 18 aldehydes) were determined to be 2.9 and 17.7, respectively (see Figure S8 for calculations), highlighting successful dendrimer formation and fluorescein ligation, with the dendrimeric structure enabling signal amplification. Further fluorescence spectroscopy analysis, showed only a negligible self-quenching effect of the fluoresceins conjugated onto the dendrimer (Figure S8).

The bioorthogonal "swarming" system was translated to the synthesis of dendrimers on the antibody Herceptin, which binds to HER2 receptors (overexpressed on cancer cells) and is widely used clinically.[61] Tri-branched aldehyde **1** (1.37 mM) was conjugated to Herceptin (137 µM) via active ester coupling giving **Her-1** as the initial focal point of the "swarm" (Figure 3 and S1). **Her-1** (10 µM) was treated, consecutively, with the aminooxyl building block **2** (to give **Her-2**, Figure S2), tetrazine **3** (to give **Her-3**, Figure S2), and fluorescein hydrazide **8** to give the bioorthogonally "swarmed" antibody **Her-4** (Figure S3), with the isolated products from each step characterized by gel electrophoresis (SDS-PAGE). The bands in the higher molecular weight region indicated the successful conjugation of the building blocks, while the fluorescent signals indicated the specificity of the fluorophore ligation (Figure S9).

With all the biocompatible building blocks in hand (no cytotoxicity was observed, Figure S10), "swarming" was achieved on SK-BR-3 (HER2 receptor positive) cells. The cells were incubated with **Her-1** (10 nM) for 4 h, washed and treated with the aminooxyl–norbornene **2** (150 nM) for 4 h to generate **Her-2** *in situ*. Next, the washed cells were treated with the bis-aldehyde tetrazine **3** (450 nM), with **Her-3** generated via IEDDA chemistry. After washing, fluorescein hydrazide **8** (1 µM) was added to the cells for 30 min to fluorescently label the dendrimers on cells, giving dendrimer **Her-4**. The localized "swarm" around the cells, with amplified fluorescence labelling (~18 fluorophores per dendrimer) was analyzed by confocal microscopy and flow cytometry (Figure 3 and S11). The cells showed 1.3-fold and 4.6-fold increase in fluorescence for cells bearing **Her-1** and **Her-4**, respectively, compared to cells treated with fluorescein conjugated Herceptin **FAM-Her** (Figure 3c). Compared to

Scheme 1. "Swarming" around a Cy5 fluorophore.[a]



[a]Compound **9** (10 μM) was treated with **2** (150 μM), **3** (450 μM) and **8** (1 μM), consecutively (with the intermediates isolated), to achieve "swarming" and to give Cy5-labelled dendrimer **10** with multiple copies of fluorescein (represented by the green balls). The swarmed dendrimer was isolated by dialysis with a molecular weight cut off of 1000 Da. For the structures of intermediates **S11** and **S12** (second- and third-generation dendrimers), see Figure S6.

untreated cells, **Her-4** gave a 23.2-fold increase in fluorescence. The "swarmed" antibodies showed similar cellular localizations as the small molecule labelled Herceptin **FAM-Her**, *i.e.*, in the cytoplasm and membranes, with fluorescence "hot spots" observed in some cells with **Her-4** (Figure 3b), which was attributed to antibody aggregation due to the increased hydrophobicity upon dendrimer modification (see Figure S12 for full image panel). No swarming, *i.e.*, dendrimer formation, was observed on HER2 negative cell line MCF-7 (Figure S13).

In conclusion, a strategy for *in situ* generation of synthetic dendrimers on live cells, using a series of consecutive bioorthogonal reactions, was pioneered. Biocompatible building blocks, with multiple bioorthogonal reactive centers(aminooxyl, aldehyde, tetrazine and norbornene), were designed and showed high reactivities and specificities for their respective reactions in biologically relevant conditions. The dendrimer structures were first synthesized in solution by "swarming" the building blocks around a Cy5 fluorophore, with the dendrimers confirmed by NMR and the amplification of fluorophores at the distal end of the swarm also shown by UV-Vis spectroscopy. The starting point (or core) of the dendrimer (carrying three reactive aldehydes) was conjugated to the clinically used antibody Herceptin, which allowed cancer cell targeting and subsequent dendrimer formation on live cells, with fluorescent signal amplification. This original "swarming" approach provides a new aspect of synthesis of non-natural chemical structures on cells and the generation of nanostructures may pave the way for the generation of artificial cellular organisms for controlling/tuning cellular behaviors.

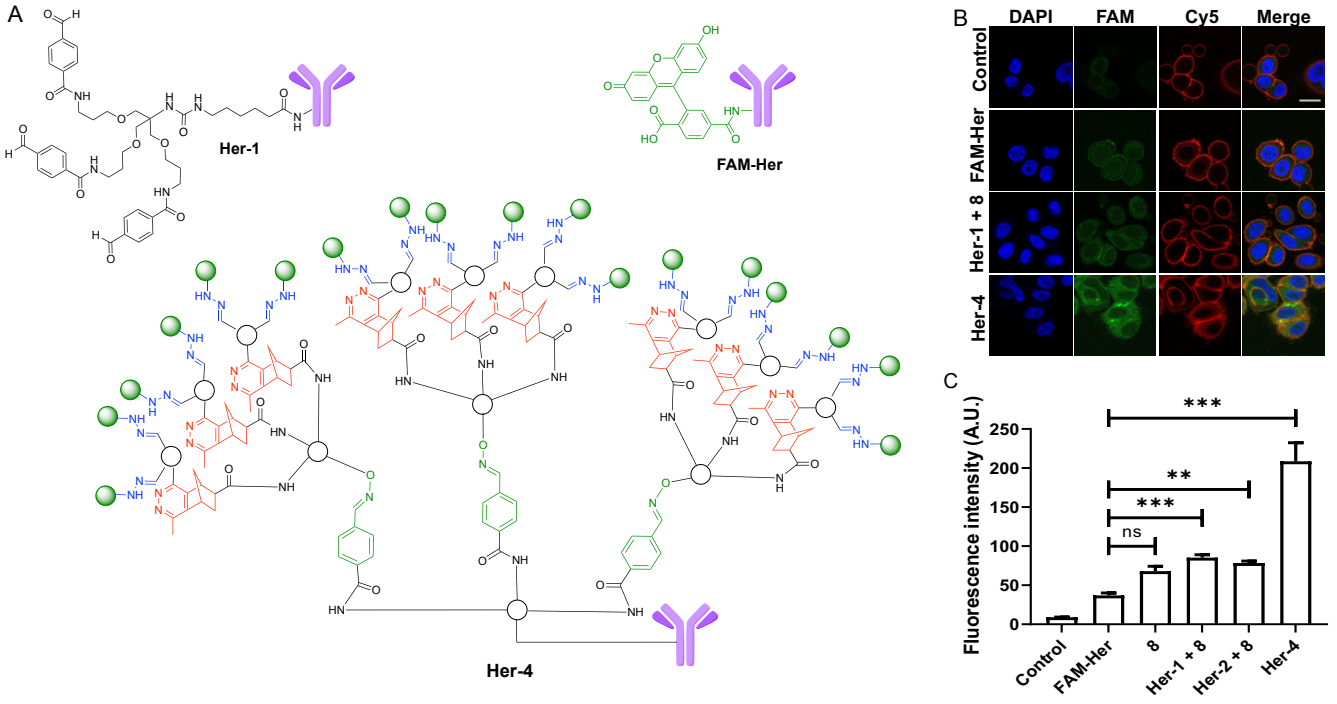


Figure 3. Dendrimer formation on live cells. (A) The structure of **FAM-Her** (fluorescein labelled control antibody) and **Her-1** (first-generation dendrimer antibody), and a schematic presentation of fluorescein (presented by the green balls) labelled **Her-4** (third-generation, for the full structure of **Her-4**, see Figure S3). One antibody modification site is shown for clarity. The bonds/branches formed via the consecutive bioorthogonal reactions are highlighted green (oxyimine formation to give **Her-2**), red (IEDDA reaction forming **Her-3**), and blue (fluorescent labelling via hydrazine ligation to give **Her-4**). (B) Confocal fluorescence microscopy images of HER2 receptor positive SK-BR-3 cells after bioorthogonal "swarming" with untreated cells as control cells (top row) and final dendrimer formation compared to small molecule labelled **FAM-Her** (10 nM, 4 h, second panel row). For dendrimer formation, the cells were treated first with **Her-1** (10 nM, 4 h), washed, and then with fluorescein hydrazide **8** (1 μM, 30 min, third row), or with **Her-1** (10 nM, 4 h) followed by building blocks **2** (150 nM, 4 h), washing, and **3** (450 nM, 30 min) to give **Her-3**, which was then labelled with **8** (1 μM, 30 min) to give **Her-4** (last row). The cell nuclei were stained with Hoechst 33342 (blue, $\lambda_{ex/em}$ = 353/483 nm), fluorescein is shown in green ($\lambda_{ex/em}$ = 490/520 nm) and the plasma membranes were stained with CellMask™ Deep Red (red, $\lambda_{ex/em}$ = 649/666 nm). Scale bar = 20 μm. (C) Fluorescence intensity (measured by flow cytometry) of untreated SK-BR-3 cells (control), cells treated with either **FAM-Her** (10 nM, 4 h), **8** (1 μM, 30 min), or "swarmed" cells treated with **Her-1** (10 nM, 4 h) followed by **8** (1 μM, 30 min) or with **Her-1** (10 nM, 4 h) followed by building blocks **2** (150 nM, 4 h), **3** (450 nM, 30 min), and **8** (1 μM, 30 min) to give **Her-4** (n = 3) ** $P < 0.01$, *** $P < 0.001$, ns (not significant) $P > 0.01$ by one-way ANOVA with Dunnett post-test, compared to the group treated with **FAM-Her**. The increase in fluorescence for the cells treated with just the fluorescent building block **8** is attributed to non-selective cellular uptake due to the high concentration used (100-fold compared to the antibody).

ASSOCIATED CONTENT

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

Supporting Schemes and Figures, and experimental procedures and copies of NMR spectra (PDF).

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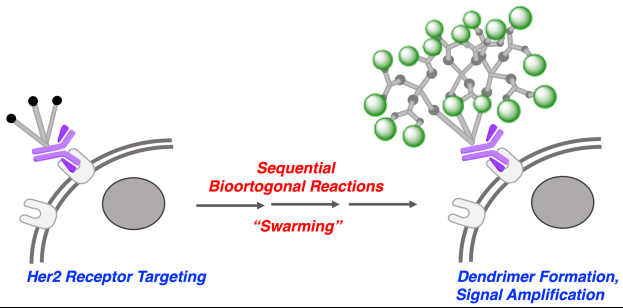
Sequential
Bioortogonal Reactions
"Swarming"
Her2 Receptor Targeting
Dendrimer Formation,
Signal Amplification