Reversible noncovalent assemblies for imaging applications

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Supramolecular host-guest interactions as a means to realize membrane-receptor specific cell surface modifications

Abstract

The application of mammalian cells for therapy is finding its way into modern medicine. However, modification or “training” of cells so that they are applicable for a specific application, remains complex. In an attempt to provide a chemical technique that enables specific, but straight-forward and generic, cellular functionalization we investigated if membrane-receptor (pre)targeting could be combined with supramolecular host-guest interactions based on β-cyclodextrin and adamantane. The feasibility of this approach was studied in cells that (over)express the chemokine receptor 4 (CXCR4) on their surface; CXCR4 plays a key role in cellular motility. CXCR4 could be targeted specifically using an adamantane (Ad)-functionalized Ac-TZ14011 peptide (guest molecule; K_D = 56 nM). To provide multivalent host molecules, fluorescent and radiolabeled β-cyclodextrin-poly(isobutylene-*alt*-maleic-anhydride)-polymers were synthesized with different fluorescent colors, number of functionalities, and lengths. The host-guest based cell-surface modifications, as well as their reversibility and effect on the cell-viability were then studied in detail. Using a second set of Ad-functionalized entities, we were able to introduce additional surface functionalities e.g. diagnostic labels. We were even able to use this last approach to drive cell-cell interactions. Combined we have shown that supramolecular interactions based on the specific targeting of a membrane receptor allows for specific and stable, yet reversible surface functionalization. Moreover, we were able to illustrate how this approach can be used to influence the cellular interaction with its surroundings.

4.1. Introduction

Cells are the cornerstones of mammalian life forms, their versatile surfaces are naturally evolved to express molecules which provide a refined means to interact with their surroundings, e.g. functionalizations.[1] These functionalities can stimulate and/or respond to cellular activity,[2] regulate vital processes such as hormone balance[3, 4] and induce immune responses.[5, 6] The strength of using cells for therapeutic purposes is progressively being recognized in medicine and is (among others) exploited in the form of immunotherapy and (stem) cell transplantations.[7-9] Such cell-based therapies often go alongside with relatively complex biological modification processes such as genetic modification[10] or metabolic tampering.[11] As each medical application desires specialized features and functions, these modification processes differ for each cell type and the pursued application. Ideally, interaction-enhancing functionalizations can be realized on different cell types using well defined and generic (chemical) functionalization approaches.[12]

By recognizing the cell surface as a (complex) chemical scaffold, one can reason that its functionality and interactions can be altered via bio-orthogonal conjugations.[13-16] Known examples are the introduction of polyelectrolyte polymers[13, 17-19] and the insertion of lipophilic anchors containing functionalities such as integrins or reactive handles.[20-24] Alternatively, one could approach cell functionalization in a way similar to the functionalization of inorganic surfaces. The opposite has been used extensively; hereby inorganic surfaces with simulated cell surfaces have been applied to mimic interactions that occur in nature.[25-27] When controllable and reversible inorganic-surface modifications are desired in aqueous environments, supramolecular host-guest interactions, e.g. using beta-cyclodextrin (β-CD) and adamantane (Ad), provide outcome.[28-30]

Membrane-expressed biomarkers provide a unique and specific fingerprint for cell populations and allow efficient and specific targeting using vectors such as antibody-derivatives and peptides.[31] Such vectors are routinely used for applications in imaging and therapy.[32] Not only can specificity be achieved by directly targeting the receptor, indirect targeting can also be applied in a pretargeting setup. Here a receptor-targeting vector is first directed towards the membrane receptor, which is then followed by a secondary functionalization, using an agent that contains e.g. a diagnostic or a therapeutic label.[33-35] Other than applying the pretargeting concept to introduce such diagnostic/therapeutic labels, the same concept could potentially also be used to introduce other functionalities such as guest moieties that provide a base for further functionalizations.

We reasoned that it would be possible to functionalize cell surfaces in a way similar to what is known for inorganic surfaces. To realize this, a combination of membrane receptor-(pre)targeting and supramolecular surface functionalization techniques were used. Herein, the chemokine receptor 4 (CXCR4),[36] a receptor that plays a key role in cellular motility as result of chemotaxis, served as the membrane-bound target. Specific functionalization of CXCR4 was achieved via the use of an adamantane functionalized Ac-TZ14011 peptide (Figure 1A (guest)). Further surface functionalization was based on the host-guest interaction between beta-cyclodextrin host molecules on fluorescent- and/or-radiolabeled β-cyclodextrin-poly(isobutylene-*alt*-maleic-anhydride)-polymers and the adamantane functionality (Figure 1B). We also illustrated how such an approach enables the introduction of additional surface functionalities (e.g. diagnostic labels) and can even be used to drive cell-cell interactions (Figure 1C).

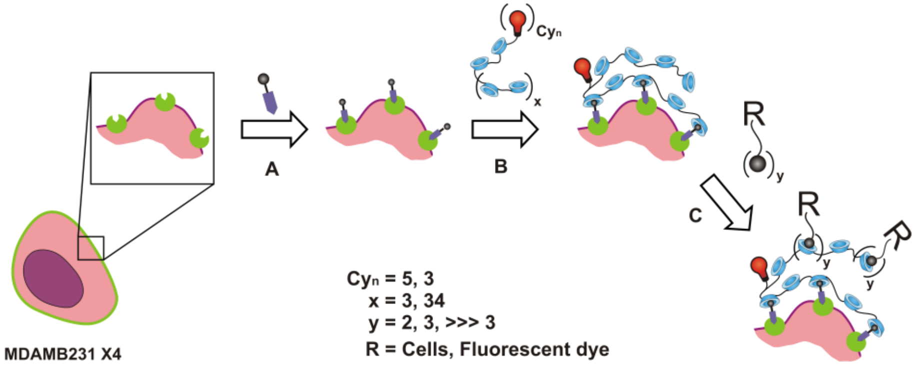

Figure 1. Schematic representation of the supramolecular functionalization of cell surfaces via targeting of the membrane-receptor CXCR4 (green). As first step, cellular specificity is introduced by using Ac-TZ14011-Ad peptide to target CXCR4 (step A). This provides an Ad-functionality on the surface that can be used as basis for more generic functionalization with β-CD polymers containing either fluorescent or radiolabels and a variable β-CD quantity (x = 3 or 34; step B). The then artificially generated CD-surfaces can be used to drive cellular interactions with entities functionalized using matching guest functionalities (step C). Hereby examples are an Ad-functionalized fluorescent dye or Ad-functionalized cells.

4.2. Results and discussion

4.2.1. Design and synthesis of the chemical components

The cyclic peptide Ac-TZ14011, a well-known targeting ligand for the CXCR4 receptor,[36] was functionalized using an Ad-group. Hereby the Ad-group pointed outwards with respect to the pharmacophore,[37] making it available for interactions with the cell's environment. Flow cytometry-based competition experiments on viable CXCR4 expressing cells (MDAMB231 X4), revealed a K_D of 56 nM for **Ac-TZ14011-Ad** (Figure AII.3), using the fluorescent Ac-TZ14011-MSAP (K_D = 187 nM) as a reference. Unmodified Ac-TZ14011 has an affinity of 8.6 nM,[38] indicating that the Ad functionality only has a moderate adverse effect on the receptor affinity.

Poly(isobutylene-*alt*-maleic-anhydride) (PIBMA) with different lengths ($PIBMA_{39}$ and $PIBMA_{389}$) were used as their anhydrides groups allow easy grafting with nucleophiles such as β-CD-NH_2, Cy3-NH_2 and Cy5-NH_2. On top, after hydrolyzing the remaining anhydrides to carboxylates, they provide good solubility in aqueous solutions (pKa_1 = 4).[39, 40] This resulted in the synthesis of two fluorescent β-CD-PIBMA-polymers and one solely fluorescent $PIBMA_{39}$-polymer without β-CD for control experiments. After conjugation, absorption spectroscopy revealed that on average 0.5 Cy5, 1.5 Cy3, and 0.4 Cy5 fluorophores were conjugated to the respective polymers. ^{1}H-NMR and NMR Diffusion Ordered Spectroscopy were used to determine the degree of CD-functionalization and to estimate the hydrodynamic diameter of the respective polymers. This yielded one polymer with 10 β-CD-units with diameter 3.2 nm (± 17.7 kDa: **$Cy5_{0.5}CD_{10}PIBMA_{39}$**), one with 72 β-CD-units with diameter 14.8 nm (± 143 kDa; **$Cy3_{1.5}CD_{72}PIBMA_{389}$**), and one without β-CD-units with diameter 3.2 nm (± 6.7 kDa; **$Cy5_{0.4}PIBMA_{39}$**).

For a more detailed description of the discussed compounds and procedures, see Appendix II.

4.2.2. Functionalization of cell surfaces

To investigate if (supramolecular) cell-surface modification via specific functionalization of the membrane-receptors was possible, CXCR4 overexpressing MDAMB231 X4 cells (including a GFP-tag attached to the CXCR4 receptor for reference) were functionalized in two steps; first with **Ac-TZ14011-Ad** (1 h; 4 °C), to allow for CXCR4 receptor targeting (Figure 1, step A) and secondly with either **$Cy5_{0.5}CD_{10}PIBMA_{39}$** or **$Cy3_{1.5}CD_{72}PIBMA_{389}$** (1 h; 4 °C) to allow further surface functionalization (Figure 1, step B). Analysis using confocal microscopy clearly indicated that the cells could be functionalized using both polymer types (Figure 2A). Furthermore, results from MTT tests performed 4 h after functionalization (Figure AII.4) showed that the cells retained their viability after functionalization with both polymers (0 - 16 µM β-CD).

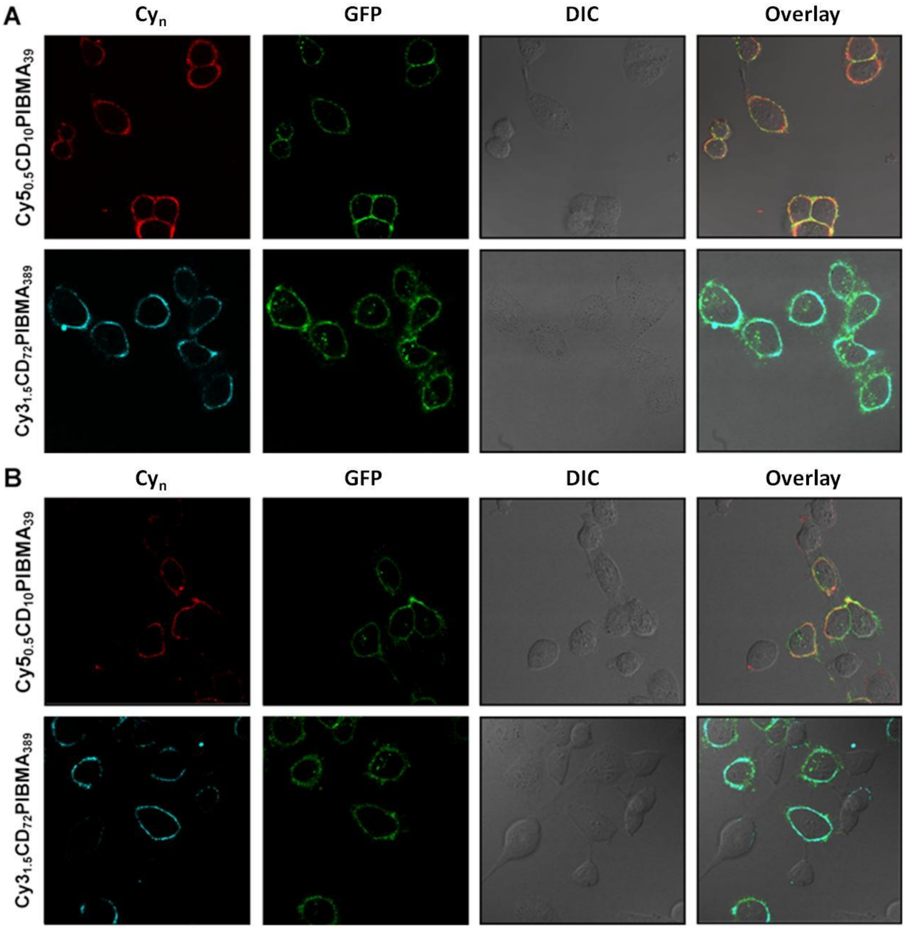

Figure 2. A) Supramolecular surface modification of MDAMB231 X4 cells with either Cy5$_{0.5}$CD$_{10}$PIBMA$_{39}$ or Cy3$_{1.5}$CD$_{72}$PIBMA$_{389}$ via specific functionalization of the membrane receptor with Ac-TZ14011-Ad. B) Mixed culture of CXCR4 overexpressing MDAMB231 X4 cells and MDAMB231 with a basal-CXCR4 expression level, after incubation with Ac-TZ14011-Ad, and subsequently with either Cy5$_{0.5}$CD$_{10}$PIBMA$_{39}$ or Cy3$_{1.5}$CD$_{72}$PIBMA$_{389}$. With Cy5 (Cy5$_{0.5}$CD$_{10}$PIBMA$_{39}$) in red, Cy3 (Cy3$_{1.5}$CD$_{72}$PIBMA$_{389}$) in blue, and GFP in green. The cell lines were discriminated based on the intrinsic GFP fluorescence signal that is only present at the MDAMB231 X4 cells leaving the MDAMB231 cells non-fluorescent. DIC = Differential interference contrast

To further study the CXCR4-receptor specificity of the functionalization process, the experiment was repeated with a mixed cell culture of vital MDAMB231 X4 (with GFP-tagged CXCR4) and as a control MDAMB231 cells (with basal CXCR4 expression and without GFP-tag). Both polymers showed the highest degree of surface functionalization on the CXCR4 overexpressing MDAMB231 X4 cells (Figure 2B), indicating receptor specificity. Confocal microscopy intensity analysis revealed that the signal intensity of **$Cy5_{0.5}CD_{10}PIBMA_{39}$** and **$Cy3_{1.5}CD_{72}PIBMA_{389}$** were respectively 5 and 8 times higher on the MDAMB231 X4 cells, compared to the signal intensity found on the cells with basal CXCR4 expression (MDAMB231).

The influence of **Ac-TZ14011-Ad** on the degree of **$Cy5_{0.5}CD_{10}PIBMA_{39}$** functionalization was examined by varying the conditions of the first incubation step as follows; 1) by omitting the use of a CXCR4-binding peptide, 2) by using non-Ad functionalized Ac-TZ14011 or 3) via the standard procedure by using **Ac-TZ14011-Ad.** Differences in **$Cy5_{0.5}CD_{10}PIBMA_{39}$** functionalization between the three set-ups were analysed using both semi-quantitative (confocal microscopy) and quantitative (flow cytometry) methods. Although the two techniques give a difference in absolute values, both depict the same trend and significance therein (Figure 3A). When **$Cy5_{0.4}PIBMA_{39}$** was used as polymer in the same set-up, this effect was not observed (Figure 3B). The latter findings indicate that β-CD—Ad interactions have an undisputable role in the functionalization process. Figure 3 further suggests that the Ac-TZ14011 peptide also interacts with β-CD. Besides hydrophobic organic guests, β-CD is known to host hydrophobic amino acids like tryptophan and tyrosine.[41, 42] When bound to the receptor, Ac-TZ14011 has one solvent exposed tyrosine (Tyr10) available for possible β-CD interactions.[36] However, from figure 3A can be concluded that the combined presence of the Ac-TZ14011 peptide and the Ad-functionality yields a significant increase in **$Cy5_{0.5}CD_{10}PIBMA_{39}$** binding. Together these findings underline the significant role that Ad- and β-CD interactions play in the functionalization process.

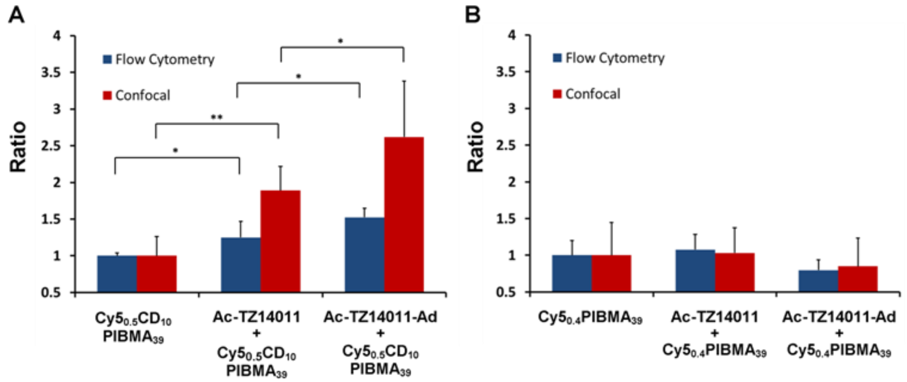

Figure 3. Normalized fluorescence intensities of A) $Cy5_{0.5}CD_{10}PIBMA_{39}$ or B) $Cy5_{0.4}PIBMA_{39}$ surface functionalized MDAMD231 X4 cells, after a first step incubation with either: no peptide, Ac-TZ14011, or Ac-TZ14011-Ad, quantified by Flow cytometry (blue) or Confocal microscopy (red). Significance of differences is marked with * ($p<0.05$) or ** ($p<0.01$).

When **Cy5-CD** was used instead of a **CD_nPIBMA_m** polymer, only a very low degree of cell functionalization could be achieved (Figure AII.5). This particular finding suggests that multivalent interactions between various β-CD-host molecules and different Ad-guest molecules are required. Based on the crystal structure of CXCR4 that was obtained from the protein data bank (PDB code 3OE0),[36] individual CXCR4 receptors have a diameter of approximately 4 to 5 nm. Although the distance between CXCR4 receptors on the membrane is unknown, it is reported that CXCR4 receptors can cluster in groups.[43, 44] Moreover, the clustering distance between CXCR4 and the membrane peptide CD4 was reported to be 5 to 10 nm.[43] When assuming a spherical structure, **$Cy5_{0.5}CD_{10}PIBMA_{39}$** has a hydrodynamic diameter of 3.2 nm in water, but when unfolded the polymer length is approximately 24 nm. Hypothetically, this should allow simultaneous interactions with multiple (clustered) CXCR4 receptors. The longer **$Cy3_{1.5}CD_{72}PIBMA_{389}$** polymer (hydrodynamic diameter = 15-19 nm; unfolded = 239 nm) should certainly allow interactions with multiple **Ac-TZ14011-Ad** functionalized CXCR4-receptors.

The strength and reversibility of the PIBMA surface modifications was monitored using radioisotope labelled **$Cy5_{0.5}CD_{10}PIBMA_{39}$** and **$Cy3_{1.5}CD_{72}PIBMA_{389}$** derivatives (see Appendix II for a detailed description of the radiolabeling procedure). In PBS, the **^{99m}Tc-$Cy5_{0.5}CD_{10}PIBMA_{39}$** and **$^{99m}Tc$-$Cy3_{1.5}CD_{72}PIBMA_{389}$** remained attached to the cell membrane for at least 1 hour. The use of either **$Cy5_{0.5}CD_{10}PIBMA_{39}$** or **$Cy3_{1.5}CD_{72}PIBMA_{389}$** as competitor resulted in a decrease in signal (Figure 4A and C). Binding of the competitors was confirmed when the experiment was performed using ^{99m}Tc-labeled competitors, indicating an exchange between the two polymers (Figure 4B and D). This effect was visually confirmed using confocal microscopy experiments that were performed over time and at the same concentrations (Figure 4E). Combined, these results suggest that the polymer functionalization is stable without the presence of a competitor, and becomes reversible when a competitor is present. The difference in length between the two polymers did not seem to influence the affinity at equal β-CD quantities.

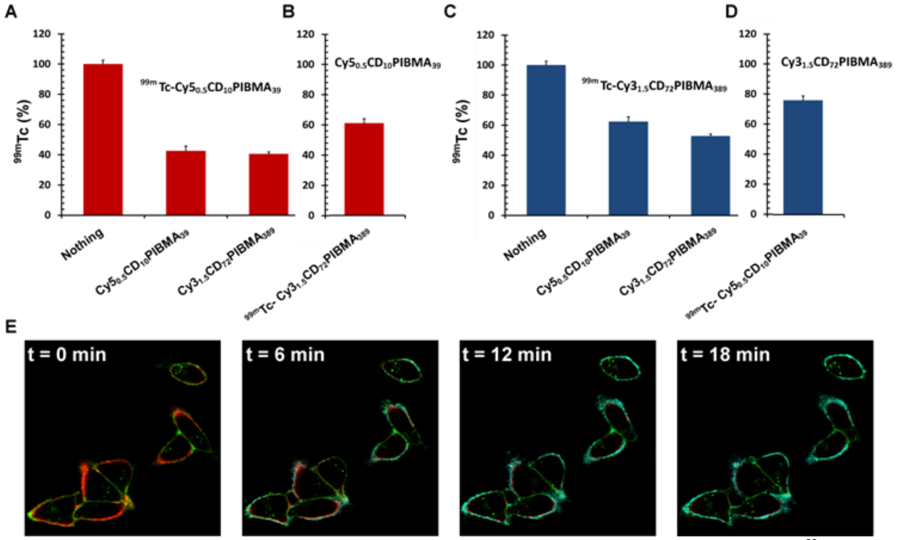

Figure 4. Competition between the polymers, quantified by radioactivity after 1 h. A) Competition of ^{99m}Tc-labeled $Cy5_{0.5}CD_{10}PIBMA_{39}$ with either $Cy5_{0.5}CD_{10}PIBMA_{39}$ or $Cy3_{1.5}CD_{72}PIBMA_{389}$, both resulting in partial replacement of the original polymer. B) $Cy5_{0.5}CD_{10}PIBMA_{39}$ functionalized cells with ^{99m}Tc-labeled $Cy3_{1.5}CD_{72}PIBMA_{389}$ as competitor, showing binding of the competitor C) Competition of ^{99m}Tc-labeled $Cy3_{1.5}CD_{72}PIBMA_{389}$ with either $Cy5_{0.5}CD_{10}PIBMA_{39}$ or $Cy3_{1.5}CD_{72}PIBMA_{389}$. D) Competition of $Cy3_{1.5}CD_{72}PIBMA_{389}$ with ^{99m}Tc-labeled $Cy5_{0.5}$-CD_{10}-PIBMA. E) Competition of $Cy5_{0.5}CD_{10}PIBMA_{39}$ with $Cy3_{1.5}CD_{72}PIBMA_{389}$, followed over time by confocal microscopy. With Cy5 ($Cy5_{0.5}CD_{10}PIBMA_{39}$) in red, Cy3 ($Cy3_{1.5}CD_{72}PIBMA_{389}$) in blue, and GFP in green.

4.2.3. Using cells as chemical scaffold for further functionalization

Since the binding of the PIBMA-polymers to the cell surface is dynamic and occurs in the presence of an excess of β-CD groups, it is therefore expected that for further functionalization there are always non-bound β-CD groups available (Figure 1, step C). This concept was initially studied using **Cy5-Ad** and **Cy5-Ad$_2$**. The monovalent **Cy5-Ad** showed very little staining of cells that were pre-functionalized with **$Cy3_{1.5}CD_{72}PIBMA_{389}$** (Figure AII.6). In contrast, the bivalent **Cy5-Ad$_2$** showed clear staining under the same conditions, providing co-localization of the CXCR4 receptor (GFP), **$Cy3_{1.5}CD_{72}PIBMA_{389}$** (Cy3), and **Cy5-Ad$_2$** (Cy5) (Figure 5A-E). Control experiments where **Cy5-Ad$_2$** was added in the absence of **$Cy3_{1.5}CD_{72}PIBMA_{389}$**, did not yield non-specific staining (Figure 5F). Again multivalency seemed to be a key component for facilitating stable interactions under *in vitro* conditions. Our finding that the β-CD functionalized cell surfaces can be used to introduce third-generation functionalizations suggests the possibility for non-covalent introduction of surface functionalities such as diagnostic labels.

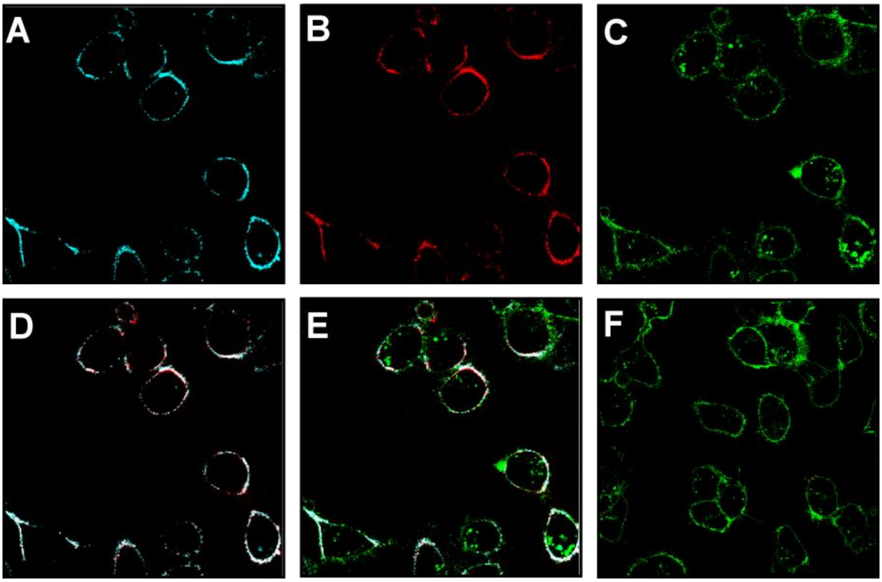

Figure 5. A-F) Confocal images of Cy5-Ad$_2$ staining on Cy3$_{1.5}$CD$_{72}$PIBMA$_{389}$ functionalized MDAMB231 X4 cells. A) Cy3 of the host-polymer in blue, B) Cy5 of bivalent guest in red, C) GFP of CXCR4 in green, D) Overlay of channels A and B, E) Overlay of channels A, B, and C, F) Cy5 (Cy5-Ad$_2$) when no polymer was added, overlay of GFP and Cy5 channels.

Given the fact that the CD-polymers interact with **Ac-TZ14011-Ad** functionalizations on the cell surface and that the secondary polymer surface functionalization enables a third-generation of surface modifications, we reasoned it would be of interest to use such technology to drive interactions between MDAMB231 X4 cells that are either functionalized with β-CD-polymers or **Ac-TZ14011-Ad**.

To study the induction of cell-cell interactions, **Ac-TZ14011-Ad** + **Cy3$_{1.5}$CD$_{72}$PIBMA$_{389}$** functionalized adhered MDAMB231 X4 cells were incubated with a solution containing **Ac-TZ14011-Ad** functionalized

MDAMB231 X4 cells in suspension (see Figure 6A for a schematic representation). The latter underwent Hoechst nucleus staining to enable discrimination and to quantify the artificially induced cell-cell interactions. After 15-30 min incubation, cell-cell interactions were quantified using confocal microscopy (Figure 6). Analysis of the obtained images revealed that on average 61% of the Hoechst stained suspended cells within the field of view showed interactions with non-Hoechst stained adherent cells.

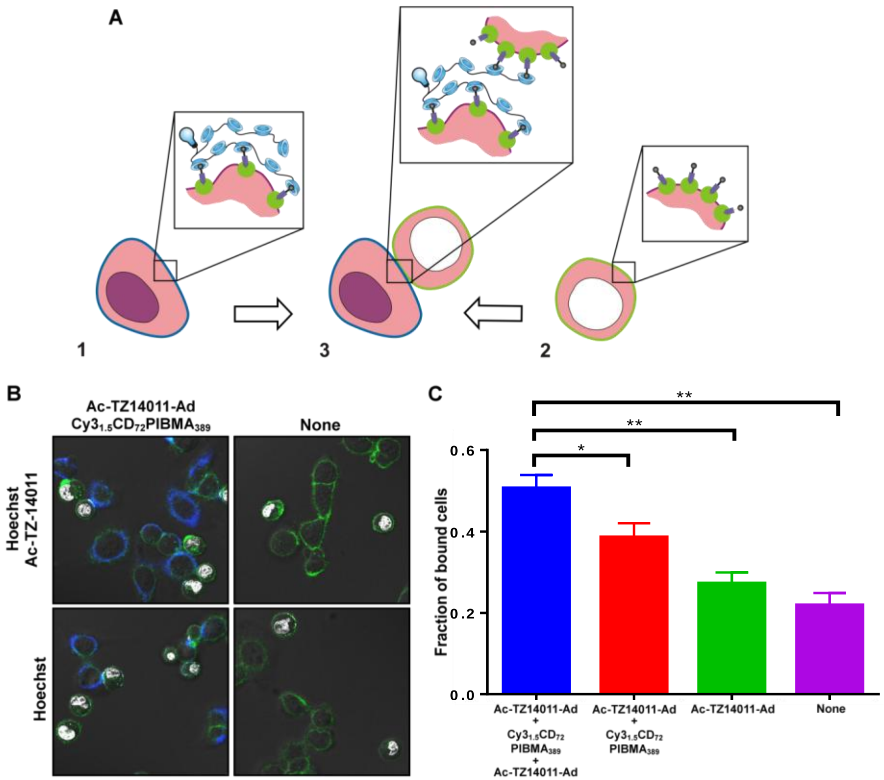

Figure 6. A) Schematic overview of inducing cell-cell interactions (3) between β-CD polymer (Cy3$_{1.5}$CD$_{10}$PIBMA$_{389}$) functionalized cells (1) and Ad (Ac-TZ14011-Ad) functionalized cells (2) with Hoechst staining (white) B) Representative confocal images of inducing supramolecular cell-cell interactions between variable functionalized MDAMB231 X4 cells. With GFP in green, Cy3 in blue and Hoechst in white. C) Average values of the fraction of cell-cell interactions in each test condition. Significance of differences is marked with * ($p<0.05$) or ** ($p<0.01$).

Control experiments where the adherent cells were not functionalized using **$Cy3_{1.5}CD_{72}PIBMA_{389}$** and/or in which the cells in suspension were not functionalized with **Ac-TZ14011-Ad** resulted in significantly lower percentages of cell-cell interactions (Figure 6C). Hence the performed functionalizations may also provide the basis for a supramolecular form of cell-cell interactions. When explored further, this approach could provide an alternative for the previously described insertion of lipophilic anchors, or genetic and metabolic tampering techniques.[10, 11]

One of the arising questions is whether the supramolecular Ac-TZ14011-Ad—CD-polymer interactions described for MDAMB231 X4 cells could also be applied to other CXCR4 expressing cell lines. To illustrate that this is indeed possible, we successfully applied the technology on a non-tumor cell type that is currently used in stem cell-therapeutics, namely the CXCR4 expressing human cardiac stem cells (Figure AII.7). Given this result, the functionalization system here presented could be expandable towards other membrane receptors expressed on other cell types of interest, using different targeting vectors, or combinations thereof.

4.3. Conclusions

This study has illustrated that targeting of membrane receptors (in this case CXCR4) can be used to achieve (reversible) supramolecular functionalizations on cell surfaces. These functionalities not only adhere to the traditional “rules” of surface modifications, they also provide a basis for the introduction of other functionalities and/or to drive cell-cell interactions.

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