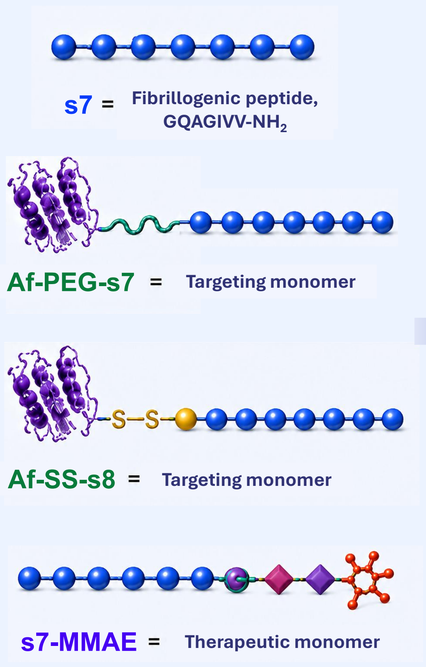
Targeting HER2-Positive Breast Cancer With a New Affibody-Nanodrug System

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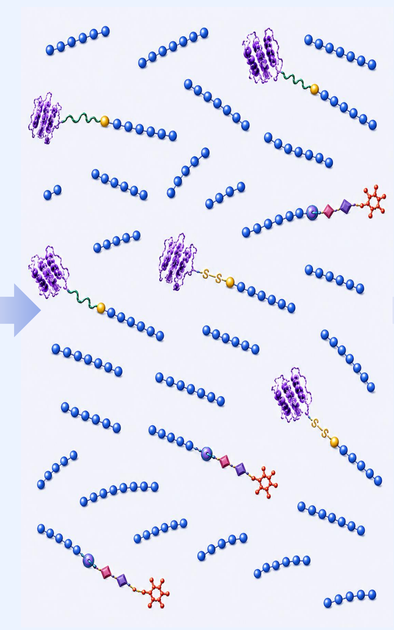
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1 Concept of the designed nanocarrier

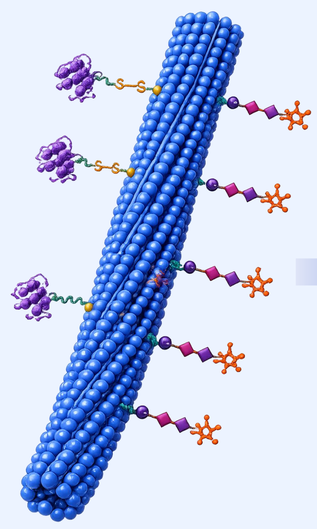
1 Modular building blocks



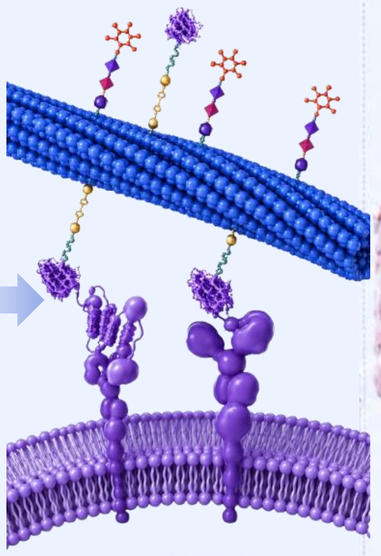
2 Fibrillization of the components at the desired ratio



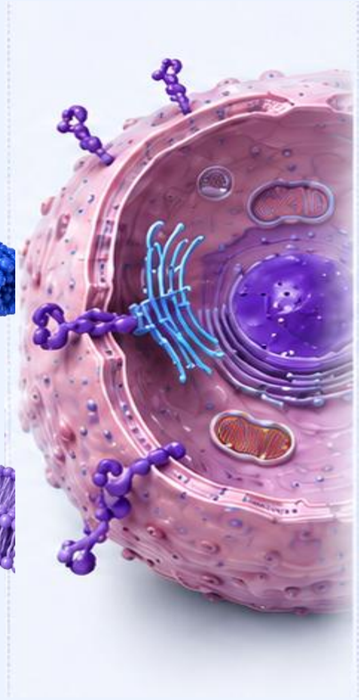
3 Hybrid peptide fibril



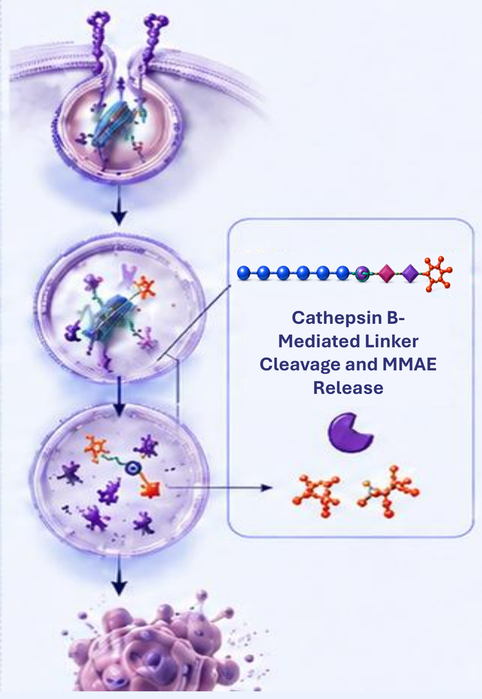
4 Targeting the HER2 receptor



5 HER2-positive cell



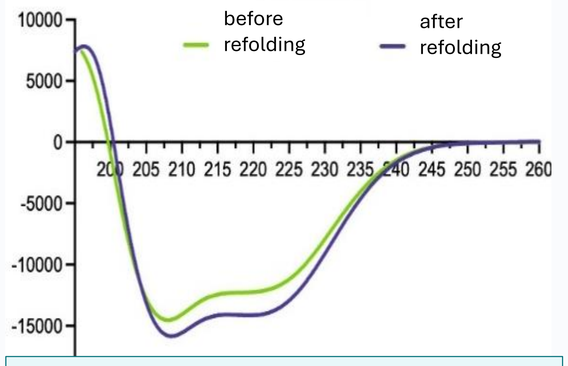
6 MMAE delivery and anticancer effect



2 Validation of the HER2-targeting module

Structural and functional evaluation of free affibody prior to incorporation into the fibrillar platform

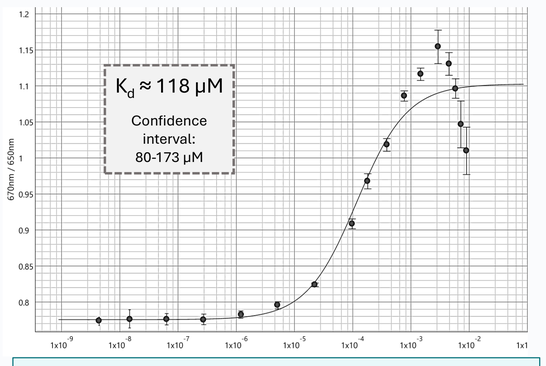
A. Circular Dichroism Spectroscopy: affibody before and after refolding



The CD spectra recorded before and after refolding were highly similar and displayed features consistent with a predominantly α-helical structure

✓ As the refolding procedure did not substantially alter the secondary-structure profile, the affibody without additional refolding was used in the HER2-binding assay

B. MST: interaction with HER2



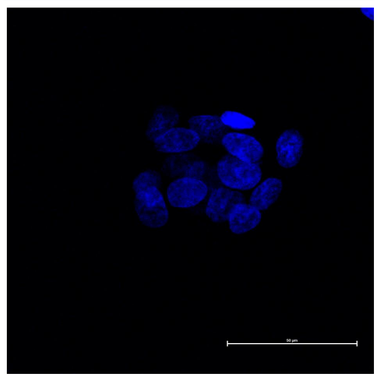
MST measurements were performed using 20 nM recombinant human HER2 and a concentration series of the affibody

✓ A concentration-dependent response was observed, supporting an interaction between the affibody and HER2. At the highest affibody concentration, the signal deviated from the expected binding profile, potentially due to self-association, aggregation, or cysteine-mediated dimerization

3 Confocal validation at the cellular interface

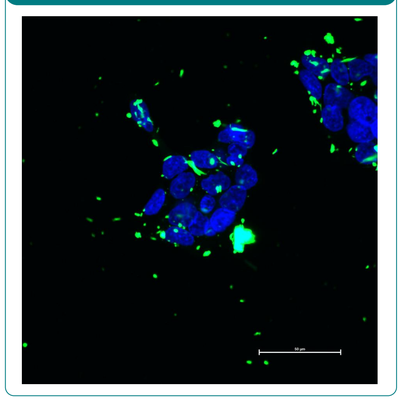
Localization of fluorescently labelled fibrils and free HER2-binding affibody relative to BT-474 cells

Control (DAPI)



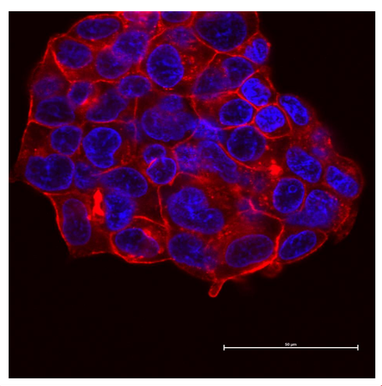
- DAPI - nuclei (blue)

Fibrils (BODIPY)



- BODIPY-labelled fibrils (green)

Affibody (Alexa Fluor 546)



- Alexa Fluor-labelled affibody (red)

BT-474 cells were cultured as a 2D monolayer in RPMI medium, allowed to adhere for 24 h, and subsequently incubated with the tested systems for 48 h. The cells were then fixed with methanol, stained with DAPI, and analysed by confocal microscopy. BODIPY-labelled fibrils and Alexa Fluor-labelled affibody were detectable in the vicinity of BT-474 cells

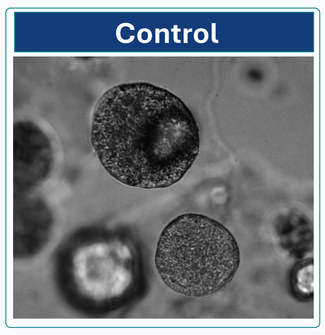
✓ Alexa Fluor-labelled affibody signal was concentrated along the cell outline, consistent with predominantly surface-associated localization

4 Biological readout

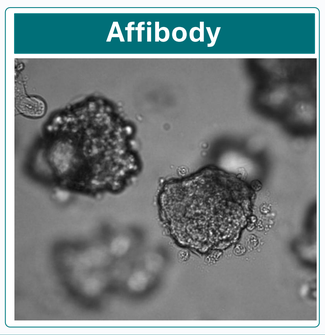
Effect of the investigated systems on BT-474 spheroids

A. Morphology

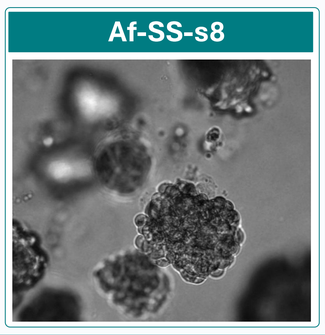
Control



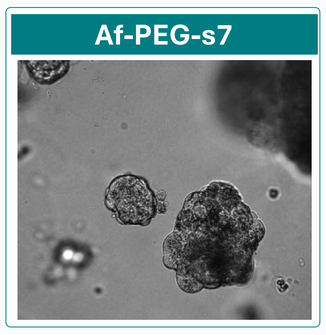
Affibody



Af-SS-s8



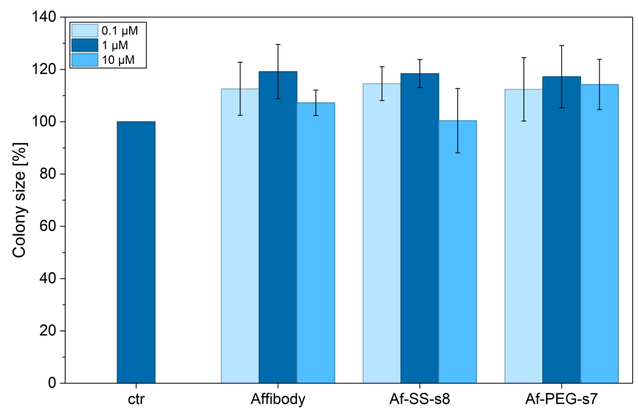
Af-PEG-s7



BT-474 spheroids were cultured in Matrigel and treated with the investigated systems (10 µM) for 19 days. Spheroid morphology was assessed by bright-field microscopy

✓ Affibody-containing systems induced visible morphological changes in BT-474 spheroids, characterized by reduced compactness and irregular borders

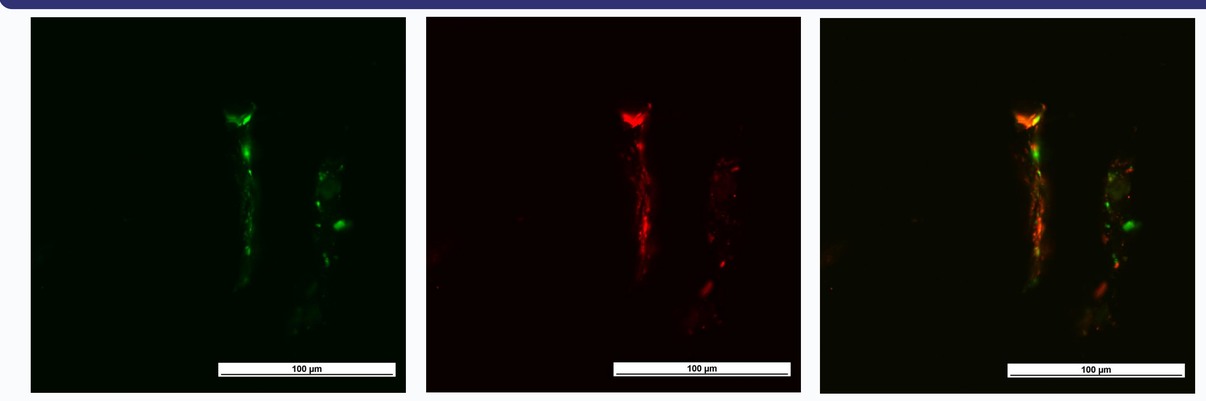
B. Colony size



BT-474 spheroids were cultured in Matrigel and treated with the investigated systems (0.1–10 µM) for 19 days. Colony size was quantified from bright-field images and expressed relative to untreated controls

✓ No reduction in BT-474 colony size was observed following treatment with affibody or affibody conjugates. Colony size remained comparable to or slightly higher than that of untreated controls across all tested concentrations

5 Affibody incorporation into fibrils



Mixed fibrillar systems composed of Alexa Fluor-labelled affibody, PyTAP-labelled s7, and unlabelled s7 (1:10:1000 ratio) were analysed by confocal microscope after 7 days of fibrillization. Numerous well-defined fibrils were visible in both fluorescence channels. The signal pattern was consistent with the co-presence of both labelled affibody and s7 within the fibrillar network

6 Conclusions

1. CD spectra demonstrated that the chemically synthesized $Z_{HER2:2891}$ affibody adopted a predominantly α-helical structure both before and after refolding
2. MST indicated a concentration-dependent interaction between the affibody and HER2, although quantitative affinity determination was limited by nonspecific effects at higher affibody concentrations
3. Confocal imaging showed fibril-associated fluorescence near BT-474 cells and predominantly surface-associated localization of the fluorescently labelled affibody
4. Affibody-containing systems altered BT-474 spheroid morphology without reducing colony size, indicating disruption of spheroid architecture rather than clear growth inhibition
5. Confocal microscopy confirmed the formation of mixed fibrils and demonstrated the incorporation of affibody and s7 within the fibrillar network

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