

Dextramabs: A Novel Format of Antibody-Drug Conjugates Featuring a Multivalent Polysaccharide Scaffold

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Antibody-drug conjugates (ADCs) are multicomponent biomolecules that have emerged as a powerful tool for targeted tumor therapy. Combining specific binding of an immunoglobulin with toxic properties of a payload, they however often suffer from poor hydrophilicity when loaded with a high amount of toxins. To address these issues simultaneously, we developed dextramabs, a novel class of hybrid antibody-drug conjugates. In these architectures, the therapeutic antibody trastuzumab is equipped with a multivalent dextran polysaccharide that enables efficient loading with a potent toxin in a controllable fashion. Our modular chemoenzymatic approach provides an access to synthetic dextramabs bearing monomethyl auristatin as releasable cytotoxic cargo. They possess high drug-to-antibody ratios, remarkable hydrophilicity, and high toxicity *in vitro*.

Having evolved from Paul Ehrlich's "magic bullet" concept, antibody-drug conjugates (ADCs) are aimed at achieving a site-specific delivery of cytotoxic agents to target cells, usually those expressing tumour-associated antigens.^[1] A three-component ADC format comprises a monoclonal antibody armed with a cytotoxic payload *via* a special, in an ideal case biodegradable, linker making use of a vast bioconjugation arsenal.^[1a,c] To date, chemistry of surface-exposed lysines or reduced cysteines at the hinge region is used to access covalent attachment of a cytotoxic counterpart. However, the lack of specificity leads to formation of heterogeneous products, which is a serious drawback regarding efficacy, immunogenicity and pharmacokinetic issues.^[1a,b,2] To overcome these deficiencies site-specific routes have been proposed, among them cysteine-^[3] and glycoengineering,^[2c,4] non-natural amino acid formats^[1b] or enzyme-mediated ligations applying transglutaminase,^[5]

sortase^[6] and formylglycine-generating enzyme,^[7] as well as the tub-tag technology.^[8]

Despite obvious progress in the field of ADCs, it is still a challenge to achieve high drug-to-antibody ratio (DAR) while maintaining hydrophilicity. Indeed, since the hydrophobic character of commonly used highly potent toxins compromises the ADC's biophysical properties, first of all solubility and aggregation, the DAR values usually do not exceed 3–4.^[1b,d,3b,9] Moreover, highly toxin-loaded ADCs possess faster clearance due to enhanced hydrophobicity.^[10] Therefore, tailoring their polarity might simultaneously enhance DAR and increase circulation time, thus modulating both efficiency and pharmacokinetics.^[11] Different approaches to address these challenges have been recently reported. Thus, Mendelsohn et al.,^[12] Lyon et al.^[11] and Santomaa et al.^[13] engineered toxic auristatin payloads towards enhanced hydrophilicity. Chen et al.^[3b] made use of thiol-ene ligation applying less hydrophobic multidrug linkers. To enhance polarity of their ADCs, Mersana Therapeutic Inc. decorated an antibody with a polymeric polyol-scaffold Fleximer[®] via hinge-region cysteines.^[14] By means of enzymatic catalysis Anami et al.^[5a] equipped a therapeutic antibody with branched PEG chains bearing numerous orthogonally addressable sites that enabled attachment of a toxic cargo in multiple copies.

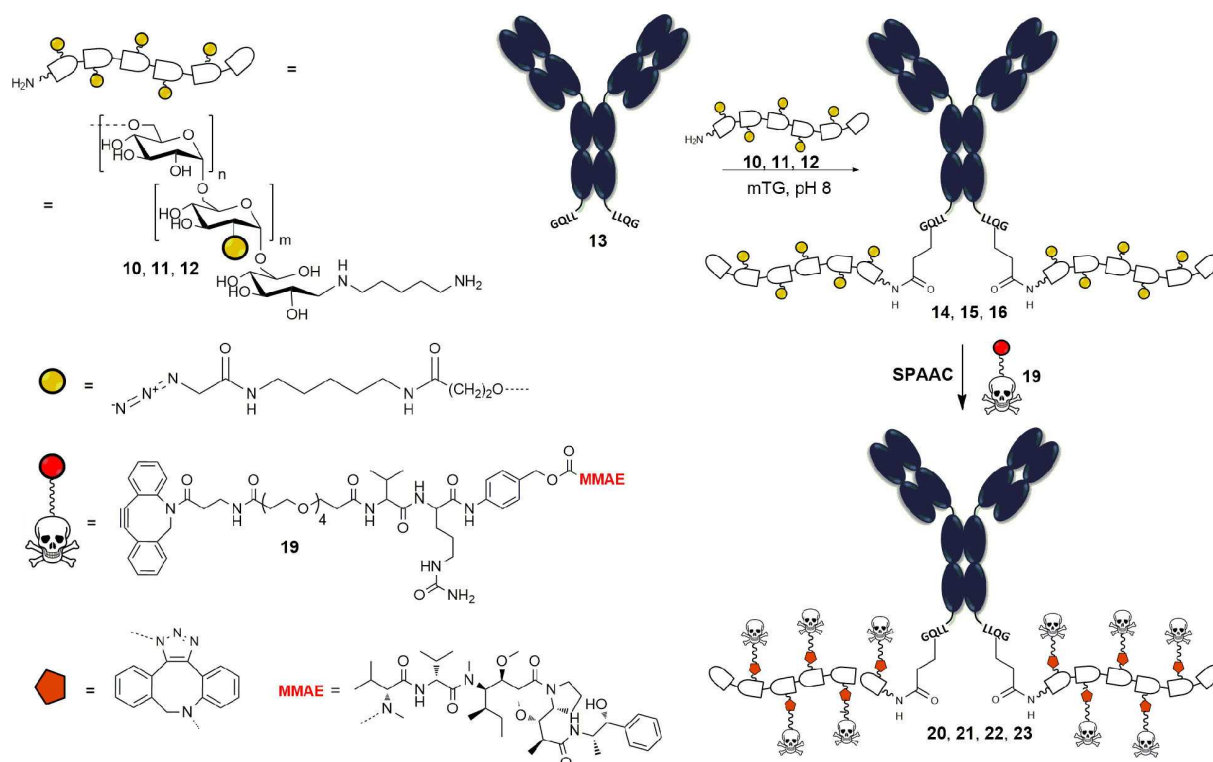
Encouraged by these achievements, we designed dextramabs, a novel ADC format comprising a therapeutic antibody as a highly specific delivery module and a hydrophilic polysaccharide scaffold carrying a releasable toxic payload in desired number of copies (Scheme 1). To enable conjugation of functional counterparts, we developed a set of efficient chemoenzymatic transformations (Scheme 1).

Dextran polysaccharide (M_w 10,000 g mol⁻¹) was chosen as a DAR- and polarity-enhancing scaffold. This biocompatible, clinically and FDA-approved glucan consisting of repeating α -(1-6)-linked oligo-D-glucose units is an accredited blood-flow enhancer and plasma volume expander.^[15] Offering certain space for chemical modifications at numerous positions, this polymer has been thoroughly investigated as a carrier for agents of diverse nature.^[15–16] A number of studies reported that conjugation to dextran positively influenced the properties of involved biomolecules.^[15,17] For example, site-specific ligation of dextran to catalase mediated by microbial transglutaminase (mTG) resulted in hybrid constructs with improved thermal stability and pharmacokinetics.^[17e] Reduced immunogenicity was demonstrated by several antibodies or Fabs when bound to dextran scaffolds.^[15,17a,b]

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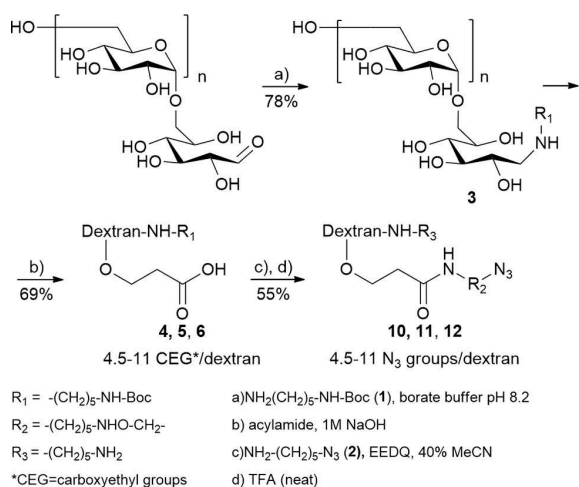


Scheme 1. General scheme for the generation of dextranabs. SPAAC: strain-promoted azide-alkyne cycloaddition. MMAE: monomethyl auristatin E.

Dextran polymer provides several possibilities for chemical modification, with oxidation of its glucose hydroxyls towards reactive aldehydes followed by coupling of a suitable nucleophile being the obvious choice.^[15,17a,b,d] Lacking selectivity, this strategy often yields conjugates with unpredicted properties, especially in view of immunoreactivity.^[15,17a,b] Therefore we decided to omit oxidation of hydroxyls making use of dextran's orthogonal addressability at its reducing end^[18] and at the glucose repeating units^[19] (Scheme 2). Thus, its sugar backbone was assigned for the

covalent immobilization of a toxic cargo, and the red-end for the enzyme-catalyzed conjugation to an antibody.

The reducing end of dextran was decorated with Boc-protected cadaverine **1** upon reductive amination, giving derivative **3** (Scheme 2) bearing a masked site for enzymatic conjugation. Afterwards, the cytotoxic payload was attached to the dextran polysaccharide backbone. The required synthetic module comprised monomethyl auristatin E (MMAE),^[20] a traceless linker (Val-Cit-PAB, Scheme 1) ensuring endosomal release of a cargo after cellular uptake, and an addressable site for the strain-promoted azide-alkyne cycloaddition (SPAAC) (Scheme 1 <xscr1). MMAE was chosen as a hydrophobic payload^[20] as it can allow to easily assess whether coupling to the dextran polymer provides advantages in terms of overall hydrophilicity. The polysaccharide scaffold was prepared for toxin attachment *via* SPAAC by decoration with an azide group, while a dibenzocyclooctyne (DBCO) moiety was introduced into the linker-toxin construct. Carboxyethylation at the C2 position of the glucose units with acrylamide followed by hydrolysis of the formed amide resulted in carboxydextrans **4**, **5**, and **6** which differed in carboxyl density (Scheme 2). The amount of carboxylic groups per dextran was controlled stoichiometrically and assessed by ¹H-NMR analysis (Section S1.1.5, ESI). We maintained this level at 4.5–11 carboxylates per dextran. The carboxyls of modified dextrans **4**, **5**, and **6** were then addressed by an amine end of the bifunctional linker **2** (Scheme 2) using EEDQ activation resulting in azide-bearing constructs **7**, **8**, and **9**. Successive removal of the Boc protecting group at the dextran reducing end yielded cadaverine-modified dextrans **10**,



Scheme 2. Synthetic route to SPAAC- and mTG-addressable dextran scaffold. EEDQ: N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

11, and 12 suitable for both SPAAC and transglutaminase-mediated chemoenzymatic bioconjugation and providing up to 11 addressable positions for the cytotoxic payload (confirmed by $^1\text{H-NMR}$ and IR, Section S1.1.6, ESI).

As a targeting/delivery module we used the monoclonal antibody trastuzumab. This immunoglobulin targets HER2-overexpressing cancer cells and is a constitutive element of the FDA-approved ADC Adcetris[®].^[1b] Trastuzumab was engineered to possess an mTG recognition tag LLQG 13 at the C-termini of its heavy chains.^[21] Transglutaminase-catalysed condensation with cadaverine-dextran towards dextran-modified antibodies 14, 15, and 16 proceeded smoothly in aqueous buffer overnight (Scheme 1, Section S1.2.1, ESI). Then the desired fluorescent/cytotoxic payload was coupled using SPAAC (Scheme 2). To that end, the cargo molecules were modified to carry a click-reactive DBCO motif (Section S1.2.2, ESI). Hydrophobic interaction chromatography (HIC) and SDS-PAGE analysis of conjugates 14, 15 and 16 revealed a dextran-to-antibody coupling efficiency between 1 and 2 (2 is the highest achievable number) indicating that every protein molecule was decorated with at least one dextran scaffold. No efforts were made yet to further enhance coupling efficiency by e.g. suppression of observed minor enzyme-mediated antibody multimerization presumably due to a reactive lysine of trastuzumab 13 (Section S1.1.8, ESI).

Following generation of dextran-trastuzumab hybrids, the toxic cargo was attached at the polysaccharide site in multiple copies. First, the fluorogenic probe DBCO-Cy5 17 was "clicked" to the azide-bearing construct 15 to yield conjugate 18 (Section S1.2.2, ESI). The completeness of SPAAC was confirmed by photometric analysis (Section S1.1.15, ESI). A well-established drug module in the context of ADC development, DBCO-PEG₃-ValCit-PAB-MMAE 19 (Scheme 1), was chosen as a payload to be "clicked" to conjugates 14, 15, and 16. Depending on the scaffold-to-payload ratio that was stoichiometrically controlled, dextramabs 20, 21, 22, and 23 were generated that differed in their DAR estimated as 2, 4, 8.5, and 11 per construct, respectively (Table S4, ESI).

To examine how the conjugation with a hydrophobic toxin influenced the polarity of synthetic dextramabs, HIC analysis was performed (Figure 1). Compared to the ADC composed of trastuzumab 25 bearing two MMAEs and lacking a polarity-enhancing dextran moiety, all dextramabs (with DAR 2–11)

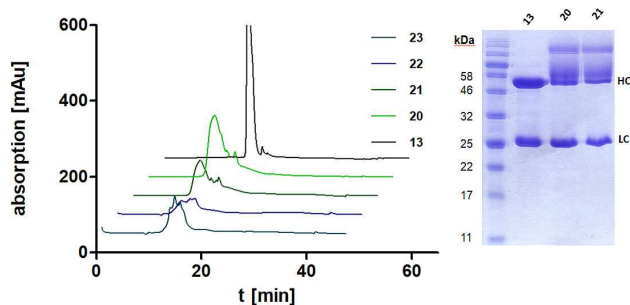


Figure 1. Left panel: HIC analysis of dextramabs 20–23 compared to the parent unmodified trastuzumab 13. Right panel: SDS PAGE of dextramabs 20 and 21 compared to solitaire antibody 13 (HC: heavy chain, LC: light chain).

showed an enormous hydrophilic shift (Section S1.1.8, ESI) indicating that the dextran hydrophilicity compensated toxin hydrophobicity even at high DAR, making these hybrid antibody-coupled dextran-toxin architectures at least as hydrophilic as the unmodified trastuzumab 13 (Figure 1).

Binding properties of synthetic dextramabs were assessed using fluorescence-activated cell sorting (FACS) on HER2-overexpressing SK-BR-3 cells. Both fluorophore- and toxin-bearing dextramabs with FAR=4 (fluorophore-to-antibody ratio) and DARs=4–8, respectively, showed binding similar to an unmodified trastuzumab. To substantiate these findings, dissociation constants (K_D) were determined by flow cytometry (Section S1.1.13–14, ESI). A K_D of 4.9 nM for the unmodified mAb 13 and a K_D of 5.9 nM for MMAE-dextramab 22 (DAR=8) was determined. Thermal shift assays of dextramabs displayed identical melting points compared to unmodified trastuzumab and therefore no loss in stability was found (S1.1.12, ESI).

The potency of four different cytotoxic dextramabs was determined *in vitro* by cell proliferation assays. The HER2-positive breast cancer cell line SK-BR-3 was chosen and CHO cells, lacking HER2, served as negative control (Figure 2, S1.1.16, ESI). A DAR-

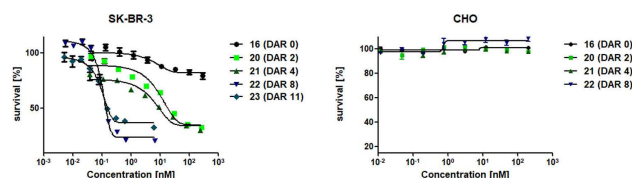


Figure 2. Cell proliferation assays. Left panel: HER 2-positive cells treated with respective dextramabs. Right panel: HER 2-negative cells treated with dextramabs. Details in text.

dependent cell killing was revealed for all examined dextramabs (20–23) on SK-BR-3 cells while no toxicity against HER2-negative cells was observed, indicating a HER2-dependent cellular uptake. Constructs 20 and 21 (DAR 2–4) showed IC_{50} values at the double-digit nanomolar range, whereas the constructs 22 and 23 (DAR 8–11) demonstrated subnanomolar inhibitory activity (IC_{50} =0.10 nM). As expected, dextramabs with no toxin warheads were found innocuous for cancer cells. Indeed, no toxicity against SK-BR-3 was observed for construct 16 (Figure 2) that lacked MMAE but possessed the highest density of enthetic azide groups. Dextramabs 22 and 23 were found more potent than a classical DAR 2 ADC 25, while possessing a highly hydrophilic character (Section S1.1.8. and S1.1.16 ESI). Nevertheless further investigations in the field of endosomal uptake of dextramabs and cathepsin-mediated cleavage of the releasable payload in dextran-bound state are needed for complete understanding of the observed cytotoxic profile.

In conclusion, we have developed a strategy towards a novel class of hybrid antibody-drug conjugates, dextramabs, which possess high toxin loading without compromising binding, stability, and solubility under physiologic conditions. In these architectures, a monoclonal antibody ensures selective binding and transport, and a polysaccharide scaffold allows for multiple, controllable attachment of a cargo. Our modular

approach includes dextran modifications at the backbone and the red-end towards site-directed enzyme-mediated monovalent conjugation with an engineered immunoglobulin of choice, followed by highly efficient SPAAC to attach a cytotoxin.

To the best of our knowledge, for the first time dextran framework was conjugated to a functional antibody site-specifically *via* its reducing end, leaving the sugar backbone intact. This has an obvious advantage compared to the common procedure that relies on random oxidation of the backbone hydroxyls with subsequent reductive amination. Though all the transformations used are rather efficient, the space for improvements still remains with respect to mTG catalysis. Engineering of improved transglutaminase recognition sites is currently ongoing in our lab.

Synthetic cytotoxic dextramabs surpass the respective ADCs in frames of hydrophilicity while possessing a high DAR, thus highlighting the potential of dextran as carrier for hydrophobic toxins. They selectively target and kill HER2-positive SK-BR-3 cells at subnanomolar concentrations showing that the dextran pendants do not affect selectivity. In a perspective, the dextramab format should allow loading of milder toxins at higher density, thus opening novel avenues to tailor-made ADCs for therapeutic applications. Follow-up animal studies will reveal, whether dextramabs with high DAR hold promise for ADCs with high potency, long *in vivo* half-life and low immunogenicity.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: ADC · bioconjugation · dextran modification · cancer · drug discovery

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