Multivalent dextran hybrids for efficient cytosolic delivery of biomolecular cargoes

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The development of novel biotherapeutics based on peptides and proteins is often limited to extracellular targets, because these molecules are not able to reach the cytosol. In recent years, several approaches were proposed to overcome this limitation. A plethora of cell-penetrating peptides (CPPs) was developed for cytoplasmic delivery of cell-impermeable cargo molecules. For many CPPs, multimerization or multicopy arrangement on a scaffold resulted in improved delivery but also higher cytotoxicity. Recently, we introduced dextran as multivalent, hydrophilic polysaccharide scaffold for multimerization of cell-targeting cargoes. Here, we investigated covalent conjugation of a CPP to dextran in multiple copies and assessed the ability of resulted molecular hybrid to enter the cytoplasm of mammalian cells without largely compromising cell viability. As a CPP, we used a novel, low-toxic cationic amphiphilic peptide L17E derived from M-lycotoxin. Here, we show that cell-penetrating properties of L17E are retained upon multivalent covalent linkage to dextran. Dextran-L17E efficiently mediated cytoplasmic translocation of an attached functional peptide and a peptide nucleic acid (PNA). Moreover, a synthetic route was established to mask the lysine side chains of L17E with a photolabile protecting group thus opening avenues for light-triggered activation of cellular uptake.

KEYWORDS
cell penetrating peptide, intracellular delivery, multimerization, oligosaccharides, peptide nucleic acids, peptides
delivery with liposomes\(^5\) and nanoparticles\(^6\) and transport with diselolenanes\(^7\) or cell-penetrating peptides (CPPs).

Comprising oligopeptides of cationic and/or amphipathic nature,\(^8\) CPPs have recently emerged as efficient vehicles ensuring cellular delivery of various cargoes, among them peptides\(^9,10\) and proteins,\(^11,12\) following different mechanisms that depend on their structural peculiarities (for the details of those mechanisms, see Ruseska and Zimmer\(^8\) and Dougherty et al.\(^13\)). Electrostatic interactions with the cellular membrane induce cell entrance of CPPs either via direct translocation or by different pathways of endocytosis, or both.\(^8\) If the endocytic uptake mechanism prevails, cargo molecules are entrapped in the endosome and prone to subsequent lysosomal degradation. As a consequence, endosomal escape is strictly required to release the cargo into the cytosol.\(^4,14\) Not only CPPs\(^15\) but also degradation. As a consequence, endosomal escape is strictly required to release the cargo into the cytosol.\(^4,14\) Not only CPPs\(^15\) but also other drug delivery systems suffer from endosomal entrapment as well\(^8,14;\) therefore, escape strategies like the use of pH-sensitive endosomal membrane-perturbing peptides have been employed to overcome this issue.\(^16–18\)

A plethora of CPPs have been developed, most of them containing a large fraction of arginines. It has been shown\(^19\) that cyclization of oligoarginines enhances direct cytoplasmic uptake; additionally, efforts were made to identify efficient CPPs by application of synthetic molecular evolution methods.\(^7\) Besides, arginine-rich cationic peptides and amphipathic and hydrophobic ones have been reported, with their uptake mechanisms remaining largely unknown\(^20\) and depending on cargo, cell types, and concentration, as well as incubation temperature and time.\(^20,21\) A subclass of these arginine-free CPPs has been recently described by Futaki and coworkers.\(^16\) Starting with M-lycotoxin, a haemolytic peptide from the wolf spider Lycosa carolinensis,\(^22\) they replaced certain amino acids at the potential hydrophobic face with glutamic acid in order to diminish the lytic activity at physiological pH by preventing hydrophobic interactions with the cellular membrane. As a result, the most promising variant L17E displayed strongly reduced cytotoxicity (more then 30-fold compared to the parent M-lycotoxin) and promoted cytoplasmic delivery of functional proteins like Cre recombinase and antibodies (IgG) upon coinubcation.\(^16\) Futaki and coworkers proposed a novel major uptake pathway by transient membrane permeabilization. Thus, interaction of L17E with the cellular membrane is thought to induce its ruffling by actin rearrangement, ultimately leading to macropinocytosis. Before the formation of the macropinosome is completed, L17E presumably ruptures the ruffled membrane, thus allowing macromolecules to directly enter the cytoplasm.\(^23\) Besides mechanistic features of cell internalization, this type of delivery peptide is particularly interesting since, due to presence of five lysines, it provides the option of chemically masking the lysine side chains using well-known photocleavable protecting groups.\(^24,25\) so that conceptually cytoplasmic delivery could be triggered by light.

Delivery of cargos via CPPs in most applications occurs via coinubcation or upon covalent linkage of the CPP to the cargo molecule.\(^26\) It has been previously demonstrated that some CPPs like arginine-rich TAT peptide or arginine-lacking transportan 10 displayed higher cellular uptake propensity when presented in multiple copies on a branched dendrimer, however, often at the cost of enhanced cytotoxicity.\(^27\) For the M-lycotoxin L17E variant (abbreviated L17E in the following), no efforts were made yet to covalently link transporter peptide and cargo, and only coinubcation experiments were described.

In this work, we intended to establish a modular approach for cytoplasmic delivery of peptides and peptide nucleic acids via conjugation to dextran polymer (Scheme 1) without largely compromising cell viability using the L17E transporter. This would allow for the easy variation of CPP-to-cargo proportion simply by adjusting their molar ratio upon dextran conjugation, aimed at finding an optimal balance between efficient cargo delivery and cytotoxicity. Indeed, nonaarginine (R9)-functionalized dextran was shown to deliver a pro-apoptotic peptide into the cytoplasm of human Jurkat cells.\(^28\) However, further analysis of the R9-dextran conjugates revealed that the constructs exhibited strongly increased cytotoxicity compared to solitary R9.\(^29\) Here, we describe the synthesis of dextran conjugates with CPP L17E and assess cytotoxicity of the hybrid construct. We examined cell-penetration with the help of dextran-L17E hybrids and observed efficient delivery of the fluorescent dye TAMRA, a GFP fluorescence complementing peptide GFP11, and a peptide nucleic acid that inhibits mRNA mis-splicing in cell nucleus resulting in restoration of GFP synthesis. We furthermore established a synthesis route towards derivatives of L17E bearing photocleavable protecting groups.

2 MATERIALS AND METHODS

2.1 Materials

All reagents and solvents were purchased from Agilent Technologies, Carbolution, Fisher Scientific, Iris Biotech, Jena Bioscience, Roth, Acros Organics, PNA Bio Inc., or Sigma Aldrich and used without further purification, unless stated otherwise. Anhydrous dimethyl sulfoxide (DMSO) and dichloromethane (DCM) were purchased from Acros Organics (Fair Lawn, NJ, USA). N,N-diisopropylethylamine (DIEA) was dried by addition of 25 g × L\(^{-1}\) calcium hydride and stirring for 3 h at reflux, followed by vacuum distillation. Boc-cadaverine, cadaverine, and dextran from Leuconstoc mesenteroides (average mol. mass 9,000–11,000; minimal branching of ~5%) were purchased from Sigma-Aldrich (Taufkirchen, Germany). From Jena Bioscience (Jena, Germany), 2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)(methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTAA) was purchased. PNA building blocks were acquired by PNA Bio Inc. (Newbury Park, CA, USA).

2.2 Solid-phase peptide synthesis

All peptides were synthesized using Fmoc-based solid-phase peptide synthesis on AmphiSpheres 40 RAM resin (0.34 mmol/g) from Agilent
Technologies (Waldbronn, Germany) or on 2-chlorotrityl chloride resin (1.6 mmol/g) from Iris Biotech (Marktredwitz, Germany). For fully automated microwave-assisted synthesis, a CEM (Kamp-Lintfort, Germany) Liberty blue® platform was applied at a 0.25 mmol scale. Coupling of amino acids was based on the N,N-disopropylcarbodiimide (DIC)/ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma, Iris-Biotech) activation system, and N-terminal Fmoc was deprotected using 20% (v/v) piperidine and 0.1 M Oxyma in N,N-dimethylformamide (DMF). As required, amino acids were protected according to a standard Fmoc/tert-butyl scheme.

When manual Fmoc-SPPS was performed, 1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, Carbolution) and N,N-diisopropylethylamine (DIEA) activation system and 20% piperidine in DMF deprotection were applied.

### 2.3 | RP-HPLC

Peptides were analyzed using either an Agilent 1100 device equipped with an Agilent Eclipse Plus RP column (C18, 100 × 4.6 mm, 3.5 μm, 95 Å) or an Agilent Infinity 1260 device equipped with an Interchim (Montluçon, France) Uptisphere Strategy RP column (C18-HQ, 3 μm, 100 × 4.6 mm) at a flow rate of 0.6 ml min⁻¹. Alternatively, analytical HPLC measurements were performed on an UltiMate 3000 system from Thermo Fisher Scientific GmbH and an ACE® (Aberdeen, Scotland) Excel® 2 C18-100 (100 × 2.1 mm, 2 μM) column at a flow rate of 0.45 ml min⁻¹ and 50°C. Linear gradients of eluent A (water +0.1% trifluoroacetic acid [TFA]) and eluent B (MeCN + 0.1% TFA) in 15 min were applied.

Peptides were purified on a semi preparative RP-HPLC Interchim Puriflash 4250 equipped with a semi preparative C18 RP column (Interchim Uptisphere Strategy; C18-HQ, 5 μm, 250 × 21.2 mm). At a flow rate of 18 ml × min⁻¹, 5 min of isocratic flow (starting concentration of eluent B) was followed by 20 min of gradient flow: eluent A, 0.1% (v/v) aq. TFA; eluent B, 90% (v/v) aq. MeCN with 0.1% (v/v) TFA. Absorption was detected at 220 and 280 nm. Or purification was performed using a JASCO (Tokyo, Japan) system (pumps PU-4086, detector UV-4075, and column oven CO-4060) with an ACE® (Aberdeen, Scotland) 5 C18-100 (150 × 21.2 mm, 5 μm, flow rate 10 ml × min⁻¹) or Ace® (Aberdeen, Scotland) 5 C18-300 (150 × 10 mm, 5 μm, flow rate 3 ml × min⁻¹) at 50°C. Aliquots of the crude peptide (10 mg) were dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (100 μl) and diluted with water (400 μl) prior to injection and filtered. Linear gradients of A (water +0.1% TFA) and B (MeOH + 0.1% TFA) in 30 min were used. Chromatograms were recorded at 215 and 280 nm.

### 2.4 | SEC-HPLC

Dextran derivatives were analyzed on a Phenomenex (Aschaffenburg, Germany) BioSpher SEC-s2000 column using an Agilent 1100 device at a flow rate of 0.6 ml × min⁻¹. Same eluents as for RP-HPLC were used, and samples were eluted at an isocratic flow of 30% eluent B.
2.5 | Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) spectra were obtained using a Shimadzu (Kyoto, Japan) LCMS-2020 mass spectrometer equipped with a Phenomenex Synergy 4 u Fusion-RP 80 (C-18, 250 × 4.6 mm, 2 μm, 80 Å). Eluent system consisted of 0.1% (v/v) aq. formic acid (FA; LC-MS grade, Fisher Scientific; Hampton, NH, USA; eluent A) and acetonitrile containing 0.1% (v/v) FA (LC-MS grade; eluent B).

Alternatively, LC-MS spectra were recorded on an UltiMate 3000 system from Thermo Fisher Scientific GmbH and an ACE® Excel® 2 C18-100 (100 × 2.1 mm, 2 μM) column at a flow rate of 0.45 ml min⁻¹ and 50°C using a linear gradient of 10% A (water +0.1% FA) to 95% B (MeCN +0.1% FA) in 15 min, coupled to an LTQ XL ion trap spectrometer from Thermo Fisher Scientific GmbH (Waltham, MA, USA). Chromatograms were monitored at wavelengths of 215, 280, and 380 nm. Electrospray ionization (ESI-HRMS) spectra were recorded on a maXis (ESI-QTOF-MS) or a micrOTOF (ESI-TOF-MS with HPLC [Agilent 1200 series]) device from Bruker Daltonik GmbH (Billerica, MA, USA).

2.6 | NMR spectroscopy

NMR measurements were recorded on an Avance III or an Avance II NMR Spectrometer at 300 MHz (Bruker BioSpin GmbH, Rheinstetten, Germany) or on a Bruker Avance Neo 600 (600 MHz). 13C NMR was performed on a Bruker Avance Neo 600 (150 MHz). Samples were dissolved in deuterium oxide, CDCl3, or DMSO-d6 from Sigma Aldrich.

2.7 | IR spectroscopy

IR measurements were performed using homogenous potassium bromide pellet or by attenuated total reflection (ATR)-IR on a FTIR-Spectrometer Spectrum Two (PerkinElmer, Rodgau, Germany). Spectra were obtained by PerkinElmer Spectrum following instructions of manufacturer: wave number area, 8,300–350 cm⁻¹; spectral solution, 0.5 cm⁻¹; wave number accuracy better than 0.01 cm⁻¹ at 3,000 cm⁻¹; wave number correctness, 0.1 cm⁻¹ at 3,000 cm⁻¹; signal-to-noise ratio, 9,300: one peak to peak, 5 s, and 32,000: one peak to peak, 1 min.

2.8 | UV/Vis spectroscopy

UV/Vis-spectroscopy was performed on a Shimadzu UVmini 1240 device using Hellma (Mülheim, Germany) Quartz SUPRASIL® cuvettes.

2.9 | Thin-layer chromatography and column chromatography

Thin-layer chromatography (TLC) analysis was performed on aluminum TLC plates with silica gel 60 F245 from Merck KGaA. Compounds were detected by fluorescence quenching with UV-light (λ = 254 nm) or via fluorescence (λex = 366 nm). Column chromatography was performed on Geduran® silica gel 60 (40–63 μm), supplied by Merck KGaA.

2.10 | Synthesis of S-3-tritylmercaptopropionic acid 25

The synthesis was performed as described in literature.30 In anhydrous DCM, 3-Mercaptopropionic acid (1 eq.) was dissolved. Trityl chloride (1.1 eq.) in anhydrous DCM was added dropwise, and the reaction was stirred overnight at ambient temperature, whereby a colorless solid precipitated. The precipitate was filtered, washed with cold diethyl ether (3 × 50 ml), and dried in airstream, yielding 7.32 g (91%).

2.11 | Synthesis of N-Boc-ethylendiamine 10

The synthesis was performed according to literature28; 1,2-ethylendiamine (1.0 eq.) was dissolved in chloroform and cooled down to 0°C. A solution of Boc-anhydride (0.1 eq.) in chloroform was added dropwise over 3 h, where after the reaction mixture was allowed to warm to room temperature overnight. The solvent was removed under reduced pressure, and the oily residue was dissolved in 2 M Na2CO3. The aqueous solution was extracted twice with DCM, and the organic phase was dried over MgSO4 and evaporated, yielding 5.13 g (81%) as colorless oil.

2.12 | Synthesis of 1-((N-Boc)-2-aminoethyl)maleimide 11

The synthesis was realized as described in literature.28 N-Boc-ethylendiamine 10 (1.0 eq.) and triethylamine (TEA) (1.45 eq.) were dissolved in diethyl ether and cooled down to 0°C. Maleic anhydride (1 eq.) dissolved in diethyl ether was added dropwise, where after the cooling bath was removed and the reaction was stirred for further 4 h. The formed brown precipitate was filtered and dissolved in acetone. TEA (2 eq.) was added, and the reaction was heated to reflux, followed by addition of acetic anhydride (1.5 eq.). The reaction was refluxed for 20 h, where after the solvent was removed by evaporation. The brown residue was purified by column chromatography (silica gel, n-hexane:ethyl acetate 1:1) to yield 4.02 g (52%) as colorless solid.

2.13 | Synthesis of N-(2-aminoethyl)maleimide 12

The synthesis was performed according to literature28; 1-((N-Boc)-2-aminoethyl)maleimide 11 was dissolved in DCM and cooled down to 0°C. TFA was added, and the reaction was stirred for 1 h while
being allowed to reach room temperature. Afterwards, the mixture was concentrated in vacuo, and the residue was precipitated in cold diethyl ether. The suspension was filtered and the residue washed with diethyl ether (4×) and dried in a desiccator. Purification by semi-preparative HPLC yielded 2.02 g (84%) was colorless solid.

### 2.14  |  Synthesis of N-(5-aminopentyl)-2-azidoacetamide 4

According to the literature, the synthesis was performed on solid support. In a syringe equipped with a frit 2-chlorotritol chloride, resin (1.6 mmol x g⁻¹, 1.0 eq.) was swollen in DCM for 20 min and washed three times with (DCM) (10 ml). A solution of cadaverine (10 eq.) and diisopropylethylamine (DIEA) (20 eq.) in DCM (5 ml) was added, and the reaction mixture was gently agitated at ambient temperature for 2 h. After washing with DCM (6 × 10 ml), a mixture of 2-azidoacetic acid (2 eq.), Oxyma (3.3 eq.) and N,N'-dicyclopropycarbodiimide (DIC) (3.3 eq.) in DCM (10 ml) was added, and the reaction mixture was gently agitated at ambient temperature for 18 h. After washing with DCM (6 × 10 ml) and diethyl ether (10 ml). The dried resin was treated with the cleavage cocktail TFA:diethylsilane (TES):H₂O:2-azidoacetamide 40 ml cold diethyl ether. Peptide was isolated by semipreparative RP-HPLC to yield 295 mg (41%) after lyophilization and analyzed with LC-ESI-MS.

### 2.15  |  Synthesis of L17E 8

L17E was synthesized via automated Fmoc-SPPS on a CEM Liberty blue® platform in 0.25 mmol scale on Amphispheres 40 RAM resin using DIC/Oxyma activation system and 20% piperidine/0.1 M Oxyma in DMF deprotection, according to the standard procedure of the manufacturer. Subsequent to final Fmoc-deprotection, the peptide resin was washed with 6 × 10 ml DCM and 2 × 5 ml diethyl ether. Side chain deprotection as well as cleavage from the solid support was performed by treating half amount of the dried resin with a mixture consisting of TFA:anisole: TES:H₂O:DTH (47:1:1:1, v:v:v:v). After shaking for 3 h at ambient temperature, the peptide was precipitated and washed with 3 × 40 ml cold diethyl ether. Peptide was isolated by semipreparative RP-HPLC to yield 9 mg (5%) after lyophilization and analyzed with LC-ESI-MS.

### 2.17  |  Synthesis of L17E-Cys 14

L17E peptide with additional C-terminal cysteine was synthesized via automated Fmoc-SPPS on a CEM Liberty blue® platform in 0.25 mmol scale on Amphispheres 40 RAM resin using DIC/Oxyma activation system and 20% piperidine/0.1 M Oxyma in DMF deprotection, according to the standard procedure of the manufacturer. Subsequent to final Fmoc-deprotection, the peptide resin was washed with 6 × 10 ml DCM and 2 × 5 ml diethyl ether. Side chain deprotection as well as cleavage from the solid support was performed by treating the dried resin with a mixture consisting of TFA:anisole: TES:H₂O:DTH (46:1:1:1, v:v:v:v). After shaking for 3 h at ambient temperature, the peptide was precipitated and washed with 3 × 40 ml cold diethyl ether. Peptide was isolated by semipreparative RP-HPLC to yield 396 mg (54%) after lyophilization and analyzed with LC-ESI-MS.

### 2.18  |  Synthesis of L17E-Pra 1

L17E peptide with additional C-terminal propargyl glycine was synthesized via manual Fmoc-SPPS in 0.25 mmol scale on Amphispheres 40 RAM resin using HATU/DIEA activation system and 20% piperidine in DMF deprotection. Coupling steps were performed under microwave irradiation (30 W, 50°C, and 10 min), and deprotection was performed at ambient temperature (5 + 10 min). After final Fmoc-deprotection, the peptide resin was washed with 6 × 10 ml DCM and 2 × 5 ml diethyl ether. Side chain deprotection as well as cleavage from the solid support was performed by treating half amount of the dried resin with a mixture consisting of TFA:anisole: TES:H₂O:2-azidoacetamide 40 ml cold diethyl ether. Peptide was isolated by semipreparative RP-HPLC to yield 90 mg (24%) after lyophilization and analyzed with LC-ESI-MS.

### 2.19  |  Synthesis of alkyne-GFP11 21

GFP11 peptide was synthesized via automated Fmoc-SPPS on a CEM Liberty blue® platform in 0.25 mmol scale on Amphispheres 40 RAM resin using DIC/Oxyma activation system and 20% piperidine/0.1 M Oxyma in DMF deprotection, according to the standard procedure of the manufacturer. After final Fmoc-deprotection, an N-terminal alkyne moiety was manually added on support, applying 4 eq. 4-pentynoic acid, 6 eq. DIC, and 6 eq. oxyma overnight. The peptide resin was washed with 6 × 10 ml DCM and 2 × 5 ml diethyl ether. Side chain deprotection as well as cleavage from the solid support was performed by treating half amount of the dried resin with a mixture consisting of TFA:anisole: TES:H₂O (47:1:1:1, v:v:v:v). After shaking for 3 h at ambient temperature, the peptide was precipitated and washed with 3 × 40 ml cold diethyl ether. Peptide was isolated by semipreparative RP-HPLC to yield 2.02 g (84%) was colorless solid.
deprotection as well as cleavage from the solid support was performed by treating the dried resin with a mixture consisting of TFA:anisole:TES:H₂O (47:1:1:1, v:v:v:v). After shaking for 3 h at ambient temperature, the peptide was precipitated and washed with 3 x 40 ml cold diethyl ether. Peptide was isolated by semipreparative RP-HPLC to yield 66 mg (12%) after lyophilization and analyzed with LC-ESI-MS.

2.20 | Synthesis of L17E-3PG 34 and L17E-5PG 35

Peptides were synthesized on H-Rink amide ChemMatrix® resin at a 0.1 mmol scale on a Liberty Blue automated microwave peptide synthesizer from CEM. Synthesis was performed according to standard Fmoc/tBu-protocol using DIC and Oxyma as coupling reagents and a solution of piperidine in DMF (20% [v/v]) for Fmoc deprotection. Attachment of the amino acid building blocks was performed as double coupling, using a modified CarboMax method (5 eq. aa, 10 eq. DIC, 5 eq. Oxyma, 0.5 eq. DIEA in DMF: 1: 75°C, 170 W, 15 s; 2: 90°C, 30 W, 110 s). Milder conditions with an elongated reaction time were applied for the coupling of His (1: 25°C, 0 W, 120 s; 2: 50°C, 35 W, 480 s). Removal of the temporary protecting group was carried out under standard conditions (1: 75°C, 260 W, 15 s; 2: 90°C, 60 W, 50 s). Manual coupling was carried out for the DEACM-protected lysine building blocks in a Discardit II™ syringe, supplied by BD and equipped with a filter by following the subsequent general procedure. To give a 0.05 M solution, 1.5 eq. of the Fmoc-protected building block were dissolved in DMF. Added to the solution were 1.48 eq. of the Fmoc-protected PNA building block, 1.45 eq. HATU and 3 eq. DIEA, which was added to the resin. The syringe was transferred to the reaction cavity of a Discover® microwave synthesizer from CEM GmbH, and coupling was performed at 50°C (25 W) for 10 min. Thereafter, the resin was washed with DMF (5 x 5 ml), and the coupling step was repeated. After completion of the peptide synthesis, the resin was washed with DMF (10 x 5 ml) and DCM (10 x 5 ml) and dried in vacuo. Acidic cleavage of the peptide from the resin and simultaneous side chain deprotection was achieved by treatment with a mixture of TFA/triisopropylsilane (TIPS)/water (95:2.5:2.5 [v/v/v]) for 3 h. The resin was washed with two additional volumes of TFA, and the combined TFA fractions were concentrated under nitrogen stream. The crude peptide was precipitated by addition of ice-cold diethyl ether (10 ml) and isolated by centrifugation and decantation of the supernatant. This procedure was repeated four times. The crude peptide was suspended in water and freeze-dried to give a fine yellow solid.

2.21 | Synthesis of TAMRA-thiol 15

TAMRA-Peg₂-Cys was synthesized via manual Fmoc-SPPS in 0.25 mmol scale on 2-chlorotrityl chloride resin. The resin was loaded with Fmoc-Cys (Trt)-OH (4 eq. + 8 eq. DIEA), next to deprotection (20% piperidine in DMF). Fmoc-8-amino-3,6-dioxoactanoic acid (Fmoc-O₂Oc-OH) (1.5 eq.) was coupled using HBTU (1.4 eq.) /DIEA (3 eq.) activation. After deprotection, the N-terminus was addressed with TAMRA-NHS (1.2 eq.) and DIEA (4 eq.), and the peptide resin was gently agitated overnight. The peptide resin was washed with 6 x 10 ml DCM and 2 x 5 ml diethyl ether. Cysteine side chain deprotection as well as cleavage from the solid support was performed by treating the dried resin with a mixture consisting of TFA:TES:H₂O (48:1:1, v:v:v). After shaking for 3 h at ambient temperature, the peptide was precipitated and washed with 3 x 40 ml cold diethyl ether. The residue was dissolved in 25% (v/v) aqueous MeCN and freeze-dried, yielding 73 mg (44%) as red solid, which was used without further purification.

2.22 | Synthesis of PNA 28

PNA was synthesized via manual Fmoc-SPPS on Amphispenspheres 40 RAM resin on a 0.05 mmol scale using HATU/DIEA activation and 20% piperidine in DMF for Fmoc-deprotection. First, Fmoc-Lys (Boc)-OH (4 eq.) was coupled using 3.95 eq. HATU and 8 eq. DIEA (2 and 3 h). Afterwards, PNA synthesis was performed using 1.5 eq. Fmoc/Bhoc protected PNA building block, 1.45 eq. HATU and 3 eq. DIEA (2 x 45 min), followed by deprotection (2 min, 2 x 3 min). After coupling of three PNA building blocks, a test cleavage was performed to analyze the success of synthesis. A spatula tip of dried resin was treated with 500 µl cleavage cocktail consisting of TFA:anisole:TES: H₂O (47:1:1:1, v:v:v:v). After shaking for 1 h, the PNA was precipitated in 1.5 ml cold diethyl ether and washed twice with 1 ml cold ether. Afterwards, the PNA was analyzed with RP-HPLC and ESI-MS. After completion of the sequence, the resin was split, and half of the batch was further modified. Trityl-protected 3-mercaptopropionic acid 25 (4 eq.) was coupled using 3.95 eq. HATU and 8 eq. DIEA for 2 h. The resin was washed with DCM (5 x 10 ml) and diethyl ether (5 x 10 ml) and dried in a desiccator overnight. Cleavage from solid support as well as side chain deprotection was performed using 5 ml of the same cleavage cocktail as for test cleavages. After shaking for 2 h, the PNA was precipitated in 40 ml cold diethyl ether and washed twice with 40 ml cold ether. After drying in a desiccator, the solid was dissolved in 5% (v/v) aqueous acetic acid and freeze-dried. A total of 32 mg (25%) was yielded as slightly yellow solid and used without further purification.

2.23 | Dextran modification

Dextran modifications, as described previously by Schneider et al., are well established in our laboratory. There is a stock of various 2-carboxyethyl (CE)-dextran-N-Boc-cadaverines, bearing different amounts of carboxy moieties, to choose from. Briefly, Dextran-N-Boc-cadaverine 1 was synthesized by reductive amination of the reducing end. Dextran (1.0 eq.), N-Boc-cadaverine (25 eq.), and NaBH₃CN (16 eq.) were dissolved in 0.05 M borate buffer pH 8.2, and the reaction mixture was stirred at 30°C for 72 h. The product
was precipitated and washed three times in cold methanol, dried, and purified using PD 10 desalting column following the instructions of the manufacturer. The product was obtained as white powder after lyophilization (95%). Successful conversion was determined by $^1$H NMR spectroscopy as described previously.31 Two-CE-dextran-N-Boc-cadaverine was synthesized by carboxylation. Dextran-N-Boc-cadaverine (1 eq.) was dissolved in 1 M NaOH, and acrylamide (2.5 eq. per desired 2-CE-group) was added. The reaction mixture was stirred at 30°C for 24 h and further 24 h at 50°C. After neutralization with 0.1 M HCl, the product was isolated using PD 10 desalting column and obtained as white powder after lyophilization. Successful conversion and quantification of carboxyethyl groups per dextran were determined by $^1$H NMR spectroscopy as described previously.31

As a starting material for further modifications, 2-CE-dextran-N-Boc-cadaverines 3, 9, 17, and 26 equipped with 6.5, 4.8, 5.4, and 10.5 2-CE-groups were employed.

Maleimide-dextran-N-Boc-cadaverines 13 and 27 were synthesized analogously to N3-dextran-N-Boc-cadaverine synthesis as reported previously.31 Two-CE-dextran-N-Boc-cadaverine 3 and 26 bearing 6.5 and 10.5 CE-groups per dextran (1.0 eq.), respectively, was dissolved in 40% aq MeCN, and EEDQ (8.5 eq. per CE-group) in 40% aq MeCN was added. After stirring at 30°C for 1 h, N-(2-aminomethyl)maleimide 12 (9 eq. per CE-group) was added and the reaction mixture stirred at 30°C for 4 h. The product was isolated using PD 10 desalting column and obtained as white powder after lyophilization (32 mg, 68% 13; 21 mg 47% 27). Successful conversion and quantification of maleimide groups per dextran were determined by $^1$H NMR spectroscopy as previously described.31

N3-dextran-N-Boc-cadaverines 6 and 18 were synthesized as reported previously.31 To that end, 2-CE-dextran-N-Boc-cadaverine bearing 4.8 and 5.4 CE-groups per dextran (1.0 eq.) was dissolved in 40% aq MeCN, and EEDQ (8.5 eq. per CE-group) in 40% aq MeCN was added. After stirring at 30°C for 1 h, N-(5-aminopentyl)-2-azidocetamide 4 (9 eq. per CE-group) was added and the reaction mixture stirred at 30°C for 4 h. The product was isolated using PD 10 desalting column and obtained as white powder after lyophilization (22 mg, 65% 6; 40 mg, 73% 18; 27 mg). Successful conversion and quantification of N3-groups per dextran were determined by $^1$H NMR spectroscopy and IR-spectroscopy as previously described.31 For Boc-deprotection, N3-dextran-N-Boc-cadaverines 6 and 18 were dissolved in TFA and stirred at ambient temperature for 30 min. The product was evaporated, redissolved in water, and obtained as white powder after lyophilization (20 mg, 89% 7; 34 mg, 86% 19; 25 mg). Quantification of deprotection was determined by $^1$H NMR spectroscopy as described previously.31

N3-dextran-TAMRA 20 was synthesized by dissolving N3-dextran-cadaverine 19 (1.0 eq.) in anhydrous DMSO. Dry DIEA (25 eq.) was added, followed by 5(6)-TAMRA-NHS (10 eq.) in anhydrous DMSO. The dark solution was shaken over night at 25°C, followed by precipitation in methanol. Afterwards, the residue was washed with MeCN, dissolved in water, and purified via PD 10 desalting column, yielding 16 mg (64%) as red solid. Purity and TAMRA loading of the product were determined by SEC-HPLC and UV/Vis spectroscopy.

### 2.24 Thiol-maleimide addition onto dextran scaffold

The respective maleimide-dextran-cadaverine 13 or 27 (1.0 eq.) was dissolved in degassed 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 6.5. A solution of (a) the respective L17E-thiol peptide 14 (1.27 eq. per maleimide group) and TAMRA-thiol 15 (0.23 eq. per maleimide group) or (b) L17E-thiol peptide (0.6 eq. per maleimide group) and PNA-thiol 28 (0.6 eq. per maleimide group) in degassed 0.1 M MES pH 6.5, 25% (v/v) MeCN was added, and the reaction mixtures stirred at ambient temperature overnight. Products were isolated using disposable PD 10 desalting columns following the instructions of the supplier and obtained as white powder after freeze drying.

### 2.25 Copper-catalyzed azide-alkyne cycloaddition on dextran

A freshly prepared aqueous solution of ascorbic acid (3.2 eq. per N3) and CuSO4·5H2O (1.6 eq. per N3) was added to the solution of L17E-alkyne 1 in 25% aq MeCN (1.6 eq. per N3). The mixture was added to the respective N3-dextran 7 (1.0 eq.) and stirred at 30°C for 3 h. Product was isolated using disposable PD 10 desalting columns following the instructions of the supplier and obtained as white powder (2.3 mg, 53%) after freeze drying.

For the synthesis of L17E-GFP11-dextran-TAMRA 22, fresh stocks of ascorbic acid and CuSO4·5H2O in water were prepared. BTTAA (6 eq. per N3) was suspended in water, and CuSO4·5H2O (1 eq. per N3) was added, yielding a dark blue solution, which decolorized after addition of ascorbic acid (3 eq. per N3). In the meantime, L17E-Pra 15 (0.8 eq. per N3) and alkyne-GFP11 21 (0.8 eq. per N3) were dissolved in DMSO and subsequently added to a solution of N3(5.4)-dextran-TAMRA 20 in sodium phosphate buffer (100 mM, pH 7.5). The aqueous solution of copper (I) was also added, yielding a final mixture of 10% (v/v) DMSO in 66 mM sodium phosphate buffer. The reaction was shaken for 2 h at 37°C and afterwards purified by consecutive analytical SEC-HPLC runs. The product-containing fractions were pooled and freeze-dried, yielding 7.4 mg (33%) as red solid.

The synthesis of GFP11-dextran-TAMRA 23 was realized by preparation of fresh stocks of ascorbic acid and CuSO4·5H2O in water. BTTAA (7.2 eq. related to dextran 20) was dissolved in water and CuSO4·5H2O (1.2 eq. per dextran 20) was added, causing the solution to turn dark blue. After addition of ascorbic acid (3.6 eq. per dextran 20), the solution decolorized. In the meantime, alkyne-GFP11 21 (3.5 eq. related to dextran 20) was dissolved in DMSO and added to N3(5.4)-dextran-TAMRA 20 in sodium phosphate buffer (100 mM, pH 7.5). The aqueous solution of copper (I) was also added, yielding a final mixture of 10% (v/v) DMSO in 66 mM sodium phosphate buffer. The reaction was shaken for 2 h at 37°C and afterwards purified by consecutive analytical SEC-HPLC runs. The product containing fractions were pooled and freeze-dried, yielding 3.6 mg as red solid.
2.26 | Synthesis of DEACM-OH 31

Synthesis was carried out according to a protocol modified from Zhang et al. A solution of 7-diethylamino-4-methylcoumarin and selenium dioxide in xylene was stirred at reflux for 48 h. The reaction mixture was allowed to cool to room temperature and filtered, and the solvent was removed under reduced pressure. The residue was dissolved in methanol, and sodium borohydride was added. The solution was stirred for 4 h at room temperature, followed by addition of water and neutralization with 1 M HCl. The organic solvent was removed under reduced pressure, and the aqueous phase was extracted with DCM (5 × 100 ml). The combined organic layers were dried over MgSO4, the solvent removed in vacuo, and the crude product was purified by column chromatography (DCM → DCM/acetone 1:1) to yield 1.92 g (43%) of the title compound.

2.27 | Synthesis of DEACM-pNP 32

DEACM-OH 31 (1.0 eq.) and 4-nitrophenyl chloroformate were dissolved in DCM and cooled to 0°C. DIEA (2.0 eq.) was added, and the solution was stirred at room temperature for 12 h. The appearing precipitate was dissolved by addition of DCM, and the reaction mixture was stirred for an additional hour. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (DCM → DCM/acetone 10:1) to yield 1.53 g (53%) of the title compound.

2.28 | Synthesis of Fmoc-L-Lys (DEACM)-OH 33

DEACM-pNP 32 (1.0 eq.) suspended in DMF/DCM (1:1) was added to Fmoc-L-Lys-OH (0.97 eq.) suspended in toluene/DCM (3:2). The suspension was cooled to 0°C, and DIEA (1.0 eq.) was added. The reaction mixture was stirred for 12 h, while allowing to come to room temperature. DIEA (0.3 eq.) was added, and the solution was stirred for an additional hour. The solvents were removed under reduced pressure, and the crude product was purified by column chromatography (DMC/MeOH/AcOH 95:5:0.1). After lyophilization from dioxane, 1.12 g (87%) of the title compound was obtained.

2.29 | Cell culture

HeLa cell line was incubated under standard conditions at 37°C in a humidified incubator with 5% CO2. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich) with 10% fetal bovine serum (FBS) (Merck KGaA, Darmstadt, Germany) and 1 × penicillin/streptomycin (pen/strep) (Life technologies, Carlsbad, CA, USA). HeLa-eGFP654 cells were ordered from the UNC Tissue Culture facility (Chapel Hill, NC, USA).

2.30 | HeLa-GFP1-10

HeLa cells were seeded in a 12-well plate in a density of 5.5 × 104 cells/well and incubated for 24 h under standard conditions (in DMEM without pen/strep). The cells were transfected with pQCXIP-GFP1-10 plasmid (Addgene plasmid #68715, gifted from Yutaka Hata) using lipofectamine 2000 (Invitrogen, supplier Thermo Fisher Scientific) following the instructions of the supplier. For the selection of stably transfected cells, the cells were treated 72 h after transfection with 0.5 μg/mL puromycin. The concentration of puromycin was increased stepwise over 2 weeks to a final concentration of 1.2 μg/mL.

2.31 | Cellular uptake assay

HeLa cells were seeded in 8- or 18-well microscopy slides in a density according to manufacturer protocol and incubated for 24 h under standard conditions at 37°C in a humidified incubator with 5% CO2 in a volume DMEM (+10% FBS) corresponding to the applied slide size. Cells were treated with the constructs in DMEM (w/o FBS) for 1 h under standard conditions at 37°C in a humidified incubator with 5% CO2. Washed with phosphate-buffered saline (PBS) twice, and incubated further 3 h in DMEM (+10% FBS) at 37°C and 5% CO2. After washing with PBS, cells were fixed in 4% paraformaldehyde (15 min, ambient temperature).

For live-cell imaging of split-GFP assay, HeLa-GFP1-10 cells were seeded in eight-well microscopy slides in a density of 1.5 × 104 cells/well. After overnight incubation under standard conditions, the medium was aspirated and the cells washed twice with PBS. Serum-free DMEM was added, followed by the compounds (10 times concentrated in PBS). After incubation for 1 h, the supernatant was removed, and the cells were washed twice with PBS and further incubated for 24 h in DMEM (+10% FBS). Prior to microscopy, the medium was aspirated, and the cells were washed twice with PBS and finally covered in either PBS or Hank’s solution (0.35 g/L NaHCO3, with Ca2+ and Mg2+).

2.32 | Cell-viability assay

For assessment of cytotoxicity of construct 7 and L17E 8, HeLa cells were seeded in a 96-well plate in a density of 1.5 × 104 cells/well in a volume of 90 μl serum free DMEM. Cells were incubated for 24 h under standard conditions at 37°C in a humidified incubator with 5% CO2, and subsequently, a serial dilution of the construct in 10 μl PBS was added and cells incubated for 1 h at 37°C and 5% CO2. After further 3 h incubation in DMEM (+10% FBS) at 37°C and 5% CO2, cell viability was measured with a Tecan® (Männedorf, Switzerland) Infinite F200 Pro using CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega) following the instructions of the supplier.

Cytotoxic studies of construct 22 were performed with HeLa cells, seeded in a 96-well plate in a density of 1.0 × 105 cells/well in a
volume of 100 μl DMEM (+10% FBS). After incubation for 24 h under standard conditions, the medium was aspirated, and the cells were washed twice with PBS. A total of 90 μl serum-free DMEM were added, and a serial dilution of construct 22 in 10 μl PBS was added. After incubation for 1 h at 37°C and 5% CO₂, the supernatant was removed, and the cells were washed twice with PBS. DMEM (+10% FBS) was added, and the cells were incubated for further 24 h, followed by measuring the cell viability using CellTiter96® AQueous One Solution Cell Proliferation Assay.

2.33 | PNA mis-splicing correction assay

A total of 3 × 10⁴ HeLa-eGFP654 cells/well in DMEM with 10% FBS were seeded in a 48-well plate and incubated at 37°C in a humidified incubator with 5% CO₂ overnight. Prior to treatment with construct 29 or thiol-PNA, the medium was removed, the cells were washed with PBS, and DMEM without L-glutamine, L-cystine, and L-methionine was added. Construct 29 in different concentrations (10x concentrated in PBS, pH 6) was added, and the cells were incubated for 30 min at 37°C. After removal of the supernatant, the cells were washed with PBS and subsequently with low-pH glycine buffer (cooled to 4°C). After an additional washing step with PBS, the cells were treated with DMEM containing 10% FBS and incubated at 37°C in a humidified incubator with 5% CO₂ for 24 h. After aspiration of the medium, cells were washed with PBS and trypsinized for 10 min, where after DMEM with 10% FBS was added. Cell suspensions were transferred to a 96-well round bottom plate and centrifuged. The supernatant was discarded, and the cells were resuspended in PBS and transferred to FACS tubes.

2.34 | Fluorescence-activated cell sorting (FACS)

Cell sorting was performed using the Influx™ (BD Bioscience, Heidelberg, Germany) cell sorter.

2.35 | Microscopy

Fluorescence microscopy was performed on a Zeiss Axio Vert A1 FL (Carl Zeiss AG, Jena, Germany) equipped with Axio Cam ICM1. GFP was detected with a λ = 470 nm laser, and TAMRA was excited at λ = 540–580 nm. Confocal laser scanning microscopy was performed on a Leica TSC SP8 confocal microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

2.36 | L17E-3PG 34 and L17E-5PG 35 uncaging studies

Removal of the photocleavable protecting groups was achieved by irradiation of a 10 μM peptide solution in PBS buffer (pH = 7.0, Roche Diagnostics GmbH, Mannheim, Germany) with 10% DMSO at 405 nm in a stirrable QS semi-micro-cuvette from Hellma GmbH & Co. KG (Mülheim, Germany). As an irradiation source, an Osela inc. (Lachine, QC, Canada) Streamline Laser (405 nm, 100 mW, 70 mA) was used. Aliquots of 10 μl were taken after 0, 10, 20, 40, 60, 90, 120, 180, 300, and 600 s; diluted to 100 μl with water, and analyzed by UPLC and LC-MS.

3 | RESULTS AND DISCUSSION

3.1 | Dextran scaffold

Comprising α-(1–6) glycosidic bonds between α-glucose repeating units, dextran polysaccharide is a hydrophilic and biocompatible material. In this work, a 10 kDa dextran from the bacterium Leuconostoc mesenteroides was used, which shows only little branching of α-(1–3) linkages. Recently, our group has reported the application of dextran as multivalent scaffold for the generation of antibody-drug conjugates with high drug-to-antibody ratio and improved hydrophilic properties. In a subsequent publication, we further demonstrated the potency of this polysaccharide as multimerization platform upon induction of tumor cell apoptosis by dextran decorated with multiple receptor-binding peptides. Herein, we designed a dextran-peptide hybrid bearing multiple L17E units. In accordance with our previous research, we derivatized both the hydroxy groups of the glucose repeating units and the reducing end to yield two orthogonal conjugation sites (Scheme 1A) to ensure a modular delivery system. The approach is based on the decoration of the repeating units with cargo and CPP in multiple copies stoichiometrically, which is a viable option for the payloads of moderate size, such as peptides or small molecules. This allows for the delivery of multiple cargo molecules simultaneously (Scheme 1B), with an additional functionality placed at the reducing end. Dextran conjugates with different density of L17E peptide were synthesized applying copper-catalyzed version of Huisgen cycloaddition or relying on thiol-maleimide chemistry. Thus, for cell cytotoxicity assay, peptide L17E was assembled by SPPS, and C-terminal alkyne was introduced by Fmoc-propargylglycine, yielding L17E derivative 1 (Table 1). Respectively, dextran wasmodified at the reducing end via reductive amination with N-Boc-cadaverine, followed by carboxyethylation at the glucose repeating units. Subsequently, amide coupling of an azide linker yielded a multivalent construct 6 comprising on average 4.8 addressable azides per dextran unit. Copper-catalyzed alkyne-azide “click” yielded dextran-L17E hybrid 7 that was used to evaluate toxicity in cell-viability assays. For the evaluation of cell penetration using functional cargoes of diverse nature, conjugation was conducted upon modifications either at the reducing end of a polysaccharide or at its repeating units or by the combination of both approaches. To modify reducing end of dextran, reductive amination with N-Boc-protected cadaverine was applied, thus enabling an orthogonal coupling site for modification. All these issues will be further discussed in Section 3.3.
| Compound | Description | Abbreviation in text |
|----------|-------------|----------------------|
| **2**    |             | Dextran-N-Boc-cad    |
| **3**    |             |                      |
| **9**    |             |                      |
| **26**   |             |                      |
| **6**    |             |                      |
| **19**   |             |                      |
| **7**    |             |                      |
| **13**   |             |                      |
| **27**   |             |                      |
| **16**   |             |                      |
| **20**   |             |                      |

(Continues)
| Compound | Description | Abbreviation in text |
|----------|-------------|----------------------|
| 22       | L17E(2.7)-GFP11(2.7)-dextran-TAMRA |  |
| 23       | GFP11-dextran-TAMRA |  |
| 29       | L17E(5.3)-PNA(5.3)-dextran-N-Boc-cad |  |

**Peptides/PNA**

| Compound | Description | Abbreviation in text |
|----------|-------------|----------------------|
| 1        | H-IWLTKFLGHAAKHEAKQQLSKL-Pra-NH$_2$ | L17E-Pra |
| 8        | H-IWLTKFLGHAAKHEAKQQLSKL-NH$_2$ | L17E |
| 14       | H-IWLTKFLGHAAKHEAKQQLSKL-Cys-NH$_2$ | L17E-Cys |
| 21       | Alkyne-GFP11 |  |
| 24       | H-RDHMLHEYVNAAGIT-NH$_2$ | GFP11 |
| 28       | Thiol-PNA |  |
| 34       | H-IWLTK$^{DEACM}$FLGKHAAK$^{DEACM}$H EAKQQLSK$^{DEACM}$L-Pra-NH$_2$ | L17E-3PG |
| 35       | H-IWLTK$^{DEACM}$FLGK$^{DEACM}$HAAK$^{DEACM}$H EAK$^{DEACM}$QQLSK$^{DEACM}$L-Pra-NH$_2$ | L17E-5PG |

**Building blocks**

| Compound | Description | Abbreviation |
|----------|-------------|--------------|
| 12       | N-(2-aminoethyl) maleimide |  |

(Continues)
Assessment of toxicity

Cytotoxicity of dextran-L17E hybrid 7 and solitary L17E 8 was studied in HeLa cells. It is interesting to note that L17E, being derived from naturally haemotoxic lycotoxin, possessed rather low toxicity in the taken cell line. Indeed, similar to Akishiba et al., we did not observe a decrease in cell viability (Figure 1) when incubating HeLa cells with L17E peptide at high—up to 70 μM—concentrations. However, the hybrid module 7 bearing on average 4.8 L17E units, showed an IC50 value of approximately 10 μM, that corresponds to 48 μM solitary L17E. This roughly 1.5-fold increase in cytotoxicity could be caused by multivalency effects, but the value is still in acceptable range. The cytotoxicity of TAMRA-labeled dextran hybrids is shown in Sections S1.1.2 and S1.2.2. Investigating cell viability in the presence of the construct possessing six L17E units per dextran molecule, we found that the toxicity increased to approximately 2.5 μM with the increasing density of peptide (Figure S4). On the other hand, in good accordance with this observation, the TAMRA-labeled construct 22 bearing only 2.7 L17E per dextran revealed moderate cytotoxicity up to 20 μM (Figure S11). Moreover, it is important to mention that TAMRA labeling could induce additional cytotoxicity as has been reported previously.37

TABLE 1

| Compound | Description | Abbreviation in text |
|----------|-------------|----------------------|
| 15       | TAMRA-Thiol |                      |
| 25       | S-Trityl-3-mercaptopropionic acid |                   |
| 30       | DEACM      |                      |
| 31       | DEACM-OH   |                      |
| 32       | DEACM-pNP  |                      |
| 33       | Fmoc-L-Lys (DEACM)-OH |              |

Note. Not mentioned are synthetic intermediates, which are given in the supporting information. Experimental details are discussed in the experimental part and in supporting information.
3.3 | Delivery of payloads using synthetic dextran-peptide hybrids

3.3.1 | Synthesis of TAMRA-labeled dextran-L17E conjugate

For validation of our modular strategy, delivery of different payloads was investigated. As a model for small molecules, we labeled our L17E-dextran hybrid with the fluorophore carboxytetramethylrhodamine (TAMRA). We first evaluated the ability of a fluorescently labeled dextran biomacromolecule equipped with multiple L17E peptide units to enter HeLa cells. To that end, we decorated a 10 kDa dextran scaffold with multiple conjugation sites as reported previously. A Boc-protected cadaverine was installed at the polysaccharide reducing end upon reductive amination resulting in compound 2 bearing a protected amine conjugation site. Next, compound 2 was modified with 6.5 carboxyethyl groups on average, via carboxyethylation of the hydroxylic groups at the C2 position of the glucose repeating units, giving dextran 9. The resulting carboxylic moieties were addressed with N-(2-aminoethyl)maleimide 12 upon activation with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), giving dextran 13 equipped with 6.5 maleimide sites for subsequent conjugation of L17E peptide and TAMRA fluorophore by thiol-maleimide Michael addition.

For that purpose, an additional cysteine was introduced in the L17E sequence C-terminally, yielding peptide 14 after 9-fluorenylmethoxycarbonyl (Fmoc) SPPS. To obtain the ultimate TAMRA-labeled L17E-dextran, construct 13 was incubated with a stoichiometric mixture of 1 part of TAMRA-thiol 15 and 5.5 of L17E-Cys peptide 14 in MES buffer, resulting in dextran 16 equipped with TAMRA label and 3.8 L17E peptides on average.

3.3.2 | Delivery of TAMRA in HeLa cells

HeLa cells were treated in medium for 1 h with TAMRA-labeled dextran (10 kDa, 25 μM), TAMRA-L17E(3.8)-dextran 16 (3.13 μM), and for comparison with TAMRA-labeled dextran (10 kDa) coincubated with L17E peptide 8 (40 μM) according to Akishiba et al. After further 3 h incubation in medium only, the cellular distribution of the fluorescent constructs was analyzed by confocal laser scanning microscopy (CLSM). While cells treated with TAMRA-labeled dextran (10 kDa, 25 μM) in absence of L17E peptide only showed weak uptake of the macromolecule (Figures 2A and S1), cells coincubated with 40 μM L17E peptide, as expected, showed clearly enhanced uptake of TAMRA-labeled dextran (10 kDa, 25 μM) with signals of intense TAMRA fluorescence punctually distributed in the cytoplasm omitting the region of the nucleus (Figures 2B and S2).
with TAMRA-L17E(3.8)-dextran 16, equipped with 3.8 covalently bound L17E peptide molecules on average, showed TAMRA fluorescence spread all over the cytosol and in the nucleus, already at 3.13 μM concentration (Figures 2C and S3). This finding encouraged us to apply the dextran scaffold with multiply attached L17E peptides as uptake-mediating module for intracellular delivery of conjugated cargo.

3.3.3 | Verification of cytoplasmic delivery

It was important to find an appropriate method to verify cytoplasmic or nuclear localization of the construct and exclude possible entrapment of the modified dextran in other cell compartments such as endosomes. To this end, the split green fluorescent protein (GFP) complementation assay was employed, based on the recombination of the fragment peptide GFP 11 with cytosolic protein GFP 1–10. The structure of GFP is defined by a beta barrel consisting of 11 β strands. In a split GFP complementation assay, a nonfluorescent version missing the C-terminal beta-strand (GFP 1–10) is functionally complemented by noncovalent assembly with the 16 amino acid peptide, GFP 11. This assay has already been used for detection of cytoplasmic uptake of the GFP 11 fragment fused to CPPs, while the GFP 1–10 fragment was stably expressed in the cytosol of cells. We synthesized dextran 19 bearing an average of 5.4 azide groups and coupled TAMRA to its reducing end (construct 20). For introduction of GFP 11 and L17E, both peptides equipped with an alkyne functionality and GFP 11 with an additional GSSG linker (construct 21) were conjugated in equimolar ratio via copper-catalyzed azide-alkyne cycloaddition (CuAAC; Figure S60) to yield construct 22 (Figure 3A). As control,

![Figure 3](image-url)

**FIGURE 3** (A) Schematic depiction of dextran construct 22. (B) Live-cell CLSM images (20×) of HeLa-GFP1–10 cells treated with 15 μM construct 22, brightfield (top left), GFP-fluorescence channel (top right), and TAMRA-fluorescence channel (bottom left) and merge.
TAMRA-dextran bearing only GFP 11 peptide was also synthesized (construct 23).

Stable transfected HeLa cells, expressing GFP 1–10 in the cytosol, were either coinubated with L17E-peptide 8 and GFP 11 peptide 24 or incubated with dextran 23 as control or 22 for 1 h in medium. After further 24 h incubation in medium, cells were analyzed with CLSM and FACS (Figure S12). Compared to cells incubated with L17E and GFP 11 (Figure S9), HeLa-GFP 1–10 cells treated with construct 22 (Figures 3B and S5–S7) displayed distinctly higher GFP-fluorescence in a concentration-dependent manner. The control dextran hybrid 23 decorated only with GFP 11 shows no significant increase in GFP fluorescence (Figure S8) compared to cells treated with PBS, confirming that L17E is required for successful cellular uptake. However, since no significant difference between GFP 11 and L17E coinubcation and PBS (Figures S9 and S10) was observed, we suggest that in this case covalent conjugation of both peptides to dextran is required for cytoplasmic uptake. Since the GFP 1–10-producing HeLa cells are a heterogenous mixture of clones obtained by transfection and cell propagation, one can expect that not all of them express GFP 1–10, which may explain the finding that not all cells display functional GFP complementation (Figure 3B).

3.3.4 | Cellular uptake of PNA-loaded dextran-L17E hybrids

As a second approach to validate cytoplasmic delivery and even nuclear uptake, a mis-splicing correction assay with bioactive PNA was performed. This assay is based on HeLa cells stably transfected with enhanced GFP (eGFP) gene, interrupted by a mutated intron. As a result of this point mutation, aberrant splicing sites are activated, leading to mRNA containing remains of the intron and therefore translation of nonfunctional eGFP. Upon hybridization of an antisense oligonucleotide, for example, PNA or morpholinos, to the mutated intron, the splicing is corrected and functional eGFP is produced. As a consequence, the amount of intracellular eGFP correlates with delivered PNA to the nucleus and can be relatively quantified using flow cytometry. In a slightly different setup, based on luciferase as reporter protein, this assay has already been used to study CPP-mediated delivery of PNA.

The required 18mer PNA was synthesized via manual Fmoc-SPPS with an additional C-terminal lysine for improved solubility. For subsequent conjugation to dextran, thiol 25 was introduced at the N-terminus of the PNA strand. Dextran 27 bearing an average of 10.5 maleimide functionalities at the repeating units on average and N-Boc cadaverine on the reducing end was decorated with thiol-PNA 28 and L17E-thiol 14 in equimolar ratio via Michael addition. HeLa-eGFP654 cells were incubated with construct 29 (Figure 4A) for 30 min in serum-free medium at 37°C, followed by further incubation in serum-containing medium for 24 h. Cells were trypsinized and subsequently analyzed by FACS (Figure S13). Cells incubated with L17E-PNA(10.5)-dextran conjugate 29 displayed higher fluorescence than untreated cells even at low concentration of 2.5 μM (Figures 4B and S13). Despite its large size of approximately 54 kDa and structural complexity, construct 29 was able to enter the nucleus, and the PNA could still hybridize to the mutated intron. The cellular uptake of thiol-PNA monomer 28 was investigated in a prior experiment, where no significant shift in fluorescence was observed (Figure S13).

3.4 | Photolabile masking of lysines to address polycation problem

The polycation dilemma is a common problem which persists for all types of drug classes and delivery systems. CPPs and other drug
delivery systems utilizing their positive charge to interact with cell membranes often show promising performance in vitro, but the results in vivo may be disappointing. Independently of the administration route, whether oral or parenteral, their polycationic character is diminished by anionic mucosal membranes, serum albumin, glycosaminoglycans, and other polyanionic cell surface structures present on, for example, red blood cells, on the way to the target site. To overcome these anionic barriers, strategies to mask the cationic sites with protecting groups until the target site is reached have been developed. These include pH-, redox- or enzyme-triggered systems, in which the underlying positive charge is unleashed under the unique conditions found in the tumor microenvironment. However, these systems are often insensitive and too slow to enable any accumulation around the tumor. A better alternative may be the application of physical stimulation of photoremoveable protecting groups (PPGs) by light at the target site. Within the pool of PPGs, 7-diethylaminocoumarin (DEACM) has shown high deprotection efficiency by irradiation at 405 nm. In the present investigation, we used the highly efficient coumarin-based DEACM caging group to mask the positively charged lysine residues of L17E. The bathochromic shift introduced by the electron-donating groups at the C7 position results in an absorption maximum around 390 nm. In addition, the molecule displays a high quantum yield and an ultrafast release.

Following a procedure by Zhang et al., commercially available DEACM 30 was oxidized via Riley oxidation using SeO2 to give the corresponding aldehyde. Subsequent reduction with NaBH4 yielded alcohol 31. Activation of the alcohol moiety was achieved by conversion into anhydride 32. DEACM-pNP, with 4-nitrophenyl chloroformate, a nucleophilic attack by the ε-amino group of Fmoc-L-Lys-OH gave the desired SPPS building block 33, Fmoc-L-Lys (DEACM)-OH.

The photocaged lysine derivative was incorporated into the sequence of L17E by standard Fmoc-SPPS. Two peptides were synthesized, bearing three (L17E-3PG) and five (L17E-5PG) cages, respectively. Uncaging studies were performed by irradiation of peptide solutions (10 μM in PBS buffer at pH = 7.0 with 10% DMSO) at 405 nm and room temperature. Aliquots were analyzed by analytical RP-HPLC at 380 nm as a function of time. After 120 s, over 95% of the starting material had been consumed, and the fully deprotected L17E was identified via mass spectrometry (Figure S14). This fast and complete removal of the PPGs under irradiation was an encouraging first result. We found that protecting all five lysines with DEACM simultaneously results in a rather hydrophobic peptide, and we encountered solubility problems in PBS of the dextran polymer upon conjugation (data not shown). To date, it is unknown which of the L17E lysines are essential for cytoplasmic uptake. This issue is currently under investigation in our laboratory aimed at generating more water-soluble cell impermeable L17E variants, where only one or two lysines are DEACM protected, thus allowing for light-triggered cargo delivery.

4 | CONCLUSION

While a wealth of literature exists on the delivery of cargoes using arginine-rich peptides, comparably little is known about cellular passage relying on the arginine-lacking L17E variant of M-lycotoxin following a proposed novel uptake mechanism. Indeed, no covalent cargo conjugates of this peptide have been reported to date. We developed a hybrid delivery system based on L17E peptide and dextran polysaccharide. Decoration of dextran with multiple L17E peptides led to a versatile module able to promote cytoplasmic delivery of covalently conjugated cargoes. As known from the literature, solitary L17E peptide was able to promote cytosolic uptake of proteins, up to full-length antibodies, upon simple coinubcation with the cargo. Having introduced a covalent bond between peptide and the cargo, combined with oligomerization on a dextran scaffold, we were able to generate polymer chimera that displayed cell cytoplasmic uptake at low concentration without compromising cell viability. It remains to be elucidated, whether the proposed uptake mechanism based on membrane ruffling also prevails for dextran-coupled L17E. The cytosolic delivery, promoted by the L17E-dextran module, was validated by split-GFP complementation assay, at which a cytosolically expressed GFP 1-10 protein fragment was complemented when the delivered GFP 11 cargo reached the cytosol and was not trapped in endosomes. Moreover, the L17E-dextran module was able to deliver bioactive peptide nucleic acids into the nucleus of HeLa cells, where the cargo PNA induced mis-splicing correction resulting in enhanced GFP fluorescence. This demonstrated the potential of dextran equipped with multiple L17E peptides to promote cytosolic and even nuclear uptake. Finally, we introduced the concept of L17E CPP lysine side chain photoprotection, which might allow for light-induced-cargo delivery with possible ramifications for photodynamic therapy.

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