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A Modular Method for the High-Yield Synthesis of Site-Specific Protein–Polymer Therapeutics

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Abstract: *A versatile method is described to engineer precisely defined protein/peptide–polymer therapeutics by a modular approach that consists of three steps: 1) fusion of a protein/peptide of interest with an elastin-like polypeptide that enables facile purification and high yields; 2) installation of a clickable group at the C terminus of the recombinant protein/peptide with almost complete conversion by enzyme-mediated ligation; and 3) attachment of a polymer by a click reaction with near-quantitative conversion. We demonstrate that this modular approach is applicable to various protein/peptide drugs and used it to conjugate them to structurally diverse water-soluble polymers that prolong the plasma circulation duration of these proteins. The protein/peptide–polymer conjugates exhibited significantly improved pharmacokinetics and therapeutic effects over the native protein/peptide upon administration to mice. The studies reported here provide a facile method for the synthesis of protein/peptide–polymer conjugates for therapeutic use and other applications.*

With more than 200 approved biopharmaceuticals marketed in the United States and/or EU and many more in preclinical pipelines since the early 1980s,[1] the field of protein/peptide therapeutics is booming[2] because of their high biological activity and specificity.[3] Unfortunately, a major barrier to their clinical adoption stems from the fact that parenteral injection remains the mainstay of administering protein and peptide drugs, but it leads to rapid clearance owing to their short in vivo half-life and poor stability,[4] which in turn requires frequent injections, leading to reduced patient compliance and undesirable side effects.[5] Covalently conjugating protein and peptide drugs to "stealth polymers" that prevent opsonization of the protein and provide extended blood circulation is an effective strategy to overcome some of these limitations.[6]

The most common approach for the synthesis of protein–polymer conjugates involves reaction of semitelechelic polymers with the reactive side chains of protein/peptide residues, which is typically carried out by the separate synthesis of protein, polymer, and linker, and sequential conjugation of the three entities to create the protein/peptide–polymer conjugate.[7] Unfortunately, most such methods provide limited control of the site and grafting ratio because most proteins/peptides contain numerous chemically reactive residues, which results in heterogeneous protein/peptide–polymer conjugates with poorly controlled stoichiometry and unacceptably low biological activity.[8] The limitations of these methods are further exacerbated when the polymer to be conjugated is a "stealth polymer" because these polymers, by definition, are designed to evade interactions with proteins, so that the yield of conjugation is typically low.[9] The residue-specific incorporation of unnatural amino acids (UAAs), especially biorthogonal "clickable" residues, into recombinant proteins/peptides provides an elegant alternative to site-specifically attach polymers with high yield. However, UAAs are expensive, especially at the concentration required for genetically encoded incorporation, and the expression yield of recombinant UAA-containing proteins/peptides is typically much lower than that of the native protein, and the site of UAA incorporation affects the yield in unexpected ways that are impossible to predict, so that this method, while conceptually attractive, requires significant up-front optimization for each protein/peptide of interest.[10] Hence, methods to synthesize protein/peptide–stealth polymer conjugates are still needed that are 1) useful for protein/peptide drugs without case-by-case optimization and 2) suitable for structurally diverse polymers, and that allow 3) site-specific and 4) stoichiometric conjugation with 5) high yield.

Motivated by this rationale, we herein report a new and versatile method to synthesize precisely defined protein/peptide–polymer conjugates with site specificity, stoichiometry, and in high yield, which should be generally applicable to diverse protein and peptide drugs (Figure 1). This modular approach consists of three steps: 1) Recombinant expression of a protein/peptide of interest, which is fused to an elastin-like polypeptide (ELP), followed by an LPETG peptide that is a substrate for the transpeptidase sortase A[11] at the C terminus of the protein or peptide; 2) quantitative installation of a clickable group at the protein/peptide by canonical sortase A mediated native peptide ligation at the C terminus of the protein/peptide; and 3) attachment of a polymer by strain-promoted azide–alkyne click reaction. We show that all three steps involved in this method can be carried out in high yield to enable the conjugation of structurally diverse stealth polymers to green fluorescent protein (GFP). We further show the therapeutic utility of a zwitterionic polymer conjugate of therapeutic exendin-4, a peptide clinically used to treat type 2 diabetes.[12] The exendin–zwitterionic polymer conjugate reduced blood glucose levels for up to three days in fed mice after a single subcutaneous (s.c.) injection, which is 18 times longer than an injection of the unmodified peptide drug. We believe that this highly efficient and modular approach to synthesize precisely defined protein/peptide–

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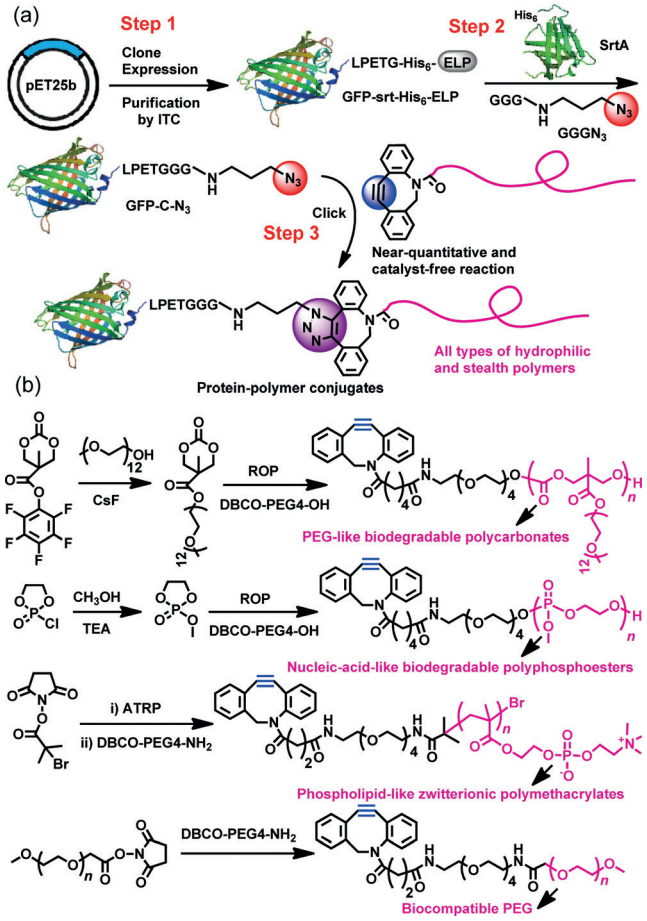

Figure 1. a) The GFP–polymer conjugates were synthesized by recombinant expression of a quaternary fusion protein, GFP-srt-His_6-ELP, followed by site-specific installation of an azido group at the C terminus of GFP by sortase A catalyzed native peptide ligation and a subsequent copper-free click reaction with DBCO-terminated polymers. b) Synthetic routes towards DBCO-terminated PEG-like biodegradable polycarbonates, nucleic-acid-like biodegradable polyphosphoesters, phospholipid-like zwitterionic polymethacrylates, and PEG.

polymer therapeutics may be applicable to a large subset of protein/peptide drugs and to diverse water-soluble polymers, and provides a general method for the clinical development of polymer conjugates as biologic drugs.

A quaternary fusion protein, GFP-srt-His_6-ELP, was recombinantly expressed to serve as the sortase A substrate (Figure 1 a); srt stands for the LPETG pentapeptide that is the recognition sequence for sortase A,[13] and ELP refers to the thermally responsive elastin-like polypeptide that is introduced to enable facile purification of the quaternary fusion protein by inverse transition cycling (ITC).[14] The LPETG peptide sequence was deliberately located between GFP and ELP, so that transpeptidation by sortase A enables the attachment of a clickable group to the C terminus of GFP and subsequent liberation of the purification tag. The GFP-srt-His_6-ELP protein was obtained in high purity with a yield of approximately 300 mg L^{-1} in a shaker-flask culture of *E. coli* transformed with an expression plasmid that encodes the fusion protein. A short linker ($GGGN_3$), consisting of an N-terminal triglycine (Gly_3) motif linked with a clickable azido group (N_3), was synthesized to serve as the nucleophile for the native peptide ligation catalyzed by sortase A[15] (see the Supporting Information, Figure S1). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the sortase A catalyzed installation of the azido group showed almost quantitative conversion after 5 h incubation at 37 °C as seen by the near-complete disappearance of the band corresponding to GFP-srt-His_6-ELP at about 67 kDa, and the appearance of two bands at approximately 28 and 39 kDa, which correspond to the azido-attached GFP (abbreviated as GFP-C-N_3) and the cleaved ELP (Figure 2 a). A control reaction was also performed using Gly_3 as the nucleophile, to yield GFP-C-Gly_3 as a negative control for the subsequent click reaction. Pure GFP-C-N_3 was easily obtained in a yield of about 85 % by immobilized metal affinity chromatography (IMAC). Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis of the purified GFP-C-N_3 showed a single peak at

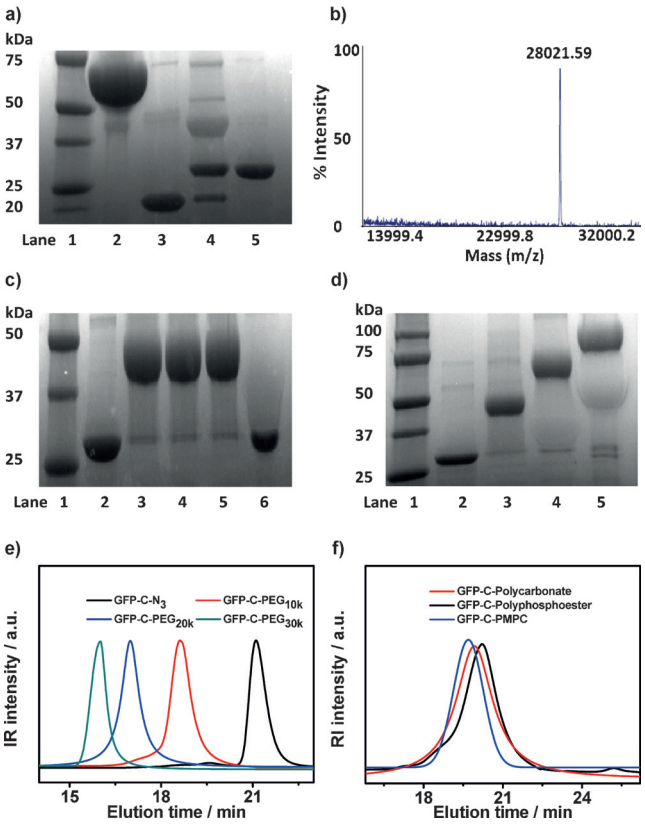

Figure 2. a) SDS-PAGE analysis of azido installation by sortase A. Lane 1: M_W marker; lane 2: GFP-srt-His_6-ELP; lane 3: sortase A; lane 4: reaction mixture after 5 h of incubation; lane 5: purified GFP-C-N_3. b) MALDI-TOF spectrum of GFP-C-N_3. c) SDS-PAGE analysis of the click reaction between GFP-C-N_3 and DBCO-PEG_{10k}. Lane 1: M_W marker; lane 2: GFP-C-N_3; lane 3–5: reaction mixture of GFP-C-N_3 and DBCO-PEG_{10k} after 10, 30, and 180 min of reaction, respectively; lane 6: mixture of GFP-C-Gly_3 and DBCO-PEG_{10k} after 180 min of reaction. d) SDS-PAGE analysis of the click reaction between GFP-C-N_3 and DBCO-PEG with different molecular weights. Lane 1: M_W marker; lane 2: GFP-C-N_3, lane 3–5: reaction mixture of GFP-C-N_3 and DBCO-PEG with molecular weights of 10, 20, and 30 kDa, respectively, after reaction for 60 min. e, f) GPC curves of GFP-C-PEG (e) and GFP-C-polycarbonate, GFP-C-polyphosphoester, and GFP-C-PMPC (f).

28021.6 Da, which closely agrees with the theoretical mass of 28034.1 Da (Error: 0.04%) for GFP-C-N_3 (Figure 2b). The Fourier transform infrared (FTIR) spectrum of GFP-C-N_3 displayed a peak at 2100 cm^{-1}, which corresponds to the stretching band of the azido group (Figure S2a), further indicating the successful attachment of the azido group to GFP. To confirm the site specificity of the azido installation, GFP-C-N_3 was subjected to trypsin digestion, and the peptide fragments were analyzed by MALDI-MS. The C-terminal peptide fragment was detected at 3796.7 Da, which is in good agreement with the theoretical mass of 3799.1 Da (Error: 0.06%), providing strong evidence that the azido group was solely attached at the C terminus of GFP (Figure S2b).

The installation of an azido group at the C terminus of a protein is a generalizable and convenient method for the site-specific attachment of structurally diverse polymers with an alkyne end group through a click reaction.[16] A set of four novel cyclooctyne-terminated water-soluble polymers, including a biodegradable polycarbonate and a polyphosphoester as well as a nondegradable polymethacrylate and polyether, were synthesized to test the generality of this approach. We chose these polymers because they are commonly used for protein conjugation. As shown in Figure 1b, the poly(ethylene glycol)-like (PEG-like) polycarbonate (DBCO-polycarbonate) and nucleic-acid-like polyphosphoester (DBCO-polyphosphoester) were synthesized by in situ organocatalyzed ring-opening polymerization (ROP) using DBCO-PEG4-OH as the initiator. Phospholipid-like zwitterionic poly[2-methacryloyloxyethyl phosphorylcholine] (DBCO-PMPC) was synthesized by atom transfer radical polymerization (ATRP) using *N*-hydroxysuccinimide (NHS) ester activated ATRP initiator and subsequent reaction with DBCO-PEG4-NH_2. PEGs (DBCO-PEG) with different molecular weights were obtained by reaction of DBCO-PEG4-amine with NHS ester terminated PEGs of different molecular weights (M_W). All of these clickable polymers were obtained with narrow polydispersity and adjustable M_W. Details of the synthesis and characterization of these polymers are described in the Supporting Information.

We next studied the click reaction by simply mixing GFP-C-N_3 with the cyclooctyne-terminated polymers at a GFP-C-N_3/polymer ratio of 1:5 in phosphate-buffered saline (PBS) without adding any catalysts and organic solvents. SDS-PAGE analysis showed that near-quantitative conversion of the reaction between GFP-C-N_3 and DBCO-PEG_{10k} was achieved only after 10 min incubation at room temperature (Figure 2c), indicating the extremely high efficiency of this catalyst-free click reaction. As expected, no reaction occurred when GFP-C-Gly_3 was added to the reaction mixture as a negative control, which suggests that the reaction occurs selectively between the azido group at the C terminus of GFP-C-N_3 and the cyclooctyne group at the chain end of the PEG, forming a site-specific (C terminus) and stoichiometric (1:1) conjugate, GFP-C-PEG. We next investigated the reaction between GFP-C-N_3 and DBCO-PEG with a range of molecular weights to understand the impact of polymer size, and potentially the steric hindrance imposed by it, on the reaction yield. As shown in Figure 2d, almost complete conversion was observed for both DBCO-PEG_{20k} and DBCO-PEG_{30k}, which suggests that at least in the M_W range of 10–30 kDa, the reaction yield does not depend on M_W, and that different M_W conjugates can be easily synthesized without the need to optimize the conjugation conditions. The quantitative conversion of GFP-C-N_3 also facilitated the purification of GFP-C-PEG with a high yield of $>80\%$, which compares favorably with the 10–20% overall yield often seen in conventional PEGylation processes.[17] Gel permeation chromatography (GPC) curves showed monomodal and symmetric elution peaks for GFP-C-PEG, which exhibited a clear shift to larger sizes with increasing M_W of the DBCO-PEG (Figure 2e). A cyclooctyne-terminated polycarbonate, polyphosphoester, and PMPC were separately conjugated to the C terminus of GFP by using identical reaction procedures (Figure 2f and Table S1).

We further investigated the blood circulation of these polymer conjugates (Figure 3a). The GFP-C-PEG and GFP-C-PMPC conjugates were chosen for pharmacokinetic studies because they are stealth polymers whose protein conjugates are known to benefit from long plasma circulation.[9] As expected, native GFP was rapidly cleared from the blood with a high total body clearance (CL) rate of 7 mL h^{-1} and a rapid terminal elimination phase ($T_{1/2\beta} = 4$ h; Table S3). In contrast, the CL rate of GFP-C-PEG decreased to 0.72 mL h^{-1}, and its $T_{1/2\beta}$ value was prolonged to 12 h. These pharmacokinetic differences resulted in a 17-fold increase in the area under the curve (AUC) of GFP-C-PEG (240% h mL^{-1}) compared to native GFP (14% h mL^{-1}). Comparable blood exposure of GFP was observed for GFP-C-PMPC with a similar hydrodynamic radius (R_h). These results reveal that C-terminal site-specific conjugation of a stealth polymer significantly improved the cumulative blood exposure of the protein.

Having established with GFP that these stealth polymer conjugates show significantly improved pharmacokinetics compared to the protein, we next investigated the utility of this method to improve the in vivo efficacy of a therapeutic peptide, exendin-4, which is a highly potent drug for the treatment of type 2 diabetes but has a notoriously short half-life of about 2 h in blood circulation.[18] Exendin-C-N_3 (Figure 3b) and two sets of exendin-C-PEG and exendin-C-PMPC conjugates with different polymer molecular weights (Table S1 and Figure S10d) but almost the same R_h were similarly synthesized and tested in vitro and in vivo.

The in vitro potencies of native exendin-4 and its conjugates were assessed by quantifying the intracellular cyclic adenosine monophosphate (cAMP) release as a result of GLP-1R activation in baby hamster kidney (BHK) cells that were stably transfected with rat GLP-1R.[19] As shown in Figure 3c, unmodified exendin-4 activates GLP-1R with a half-maximal effective concentration (EC_{50}) of 0.16 ± 0.07 nM. Grafting PEG to the C terminus of exendin-4 increased the EC_{50} value to 0.40–0.66 nM whereas the PMPC conjugates had EC_{50} values of 0.27–0.44 nM (Table S2). The slightly lower potency of the conjugates, seen by their approximately twofold greater EC_{50}, is consistent with results obtained for other polymer conjugates and due to the steric hindrance imposed by the polymer.[20]

Next, the in vivo efficacy of these conjugates was assessed in six-week-old male C57BL/6J mice that were maintained on

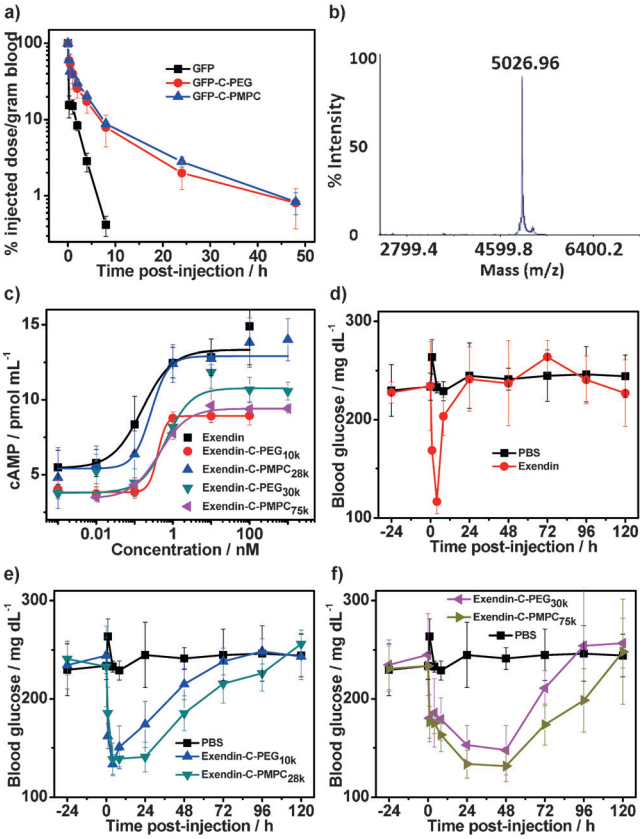

Figure 3. a) Blood concentration as a function of time post-injection. b) MALDI-TOF spectrum of exendin-C-N_3. c) cAMP response of exendin-4 conjugates in BHK cells expressing GLP-1R. Fed blood glucose levels in six-week-old male C57Bl/6J mice ($n=5$) maintained on a 60 kCal% diet before and after a single s.c. injection of d) unmodified exendin-4, e) exendin-C-PEG_{10k} and exendin-C-$PMPC_{28k}$, and f) exendin-C-PEG_{30k} and exendin-C-$PMPC_{75k}$. Exendin-4 and the conjugates were administered at a dose of 25 nmol kg^{-1}, and PBS was injected as a control at equivalent volume at $t=0$ h. The glucose levels of blood sampled from the tail of the mouse were measured at the given time points.

a 60 kCal% fat diet, so as to develop a diabetic phenotype.[21] The PEG and PMPC conjugates were administered to mice ($n=5$) by a single s.c. injection at a dose of 25 nmol kg^{-1} mouse body weight, and the fed glucose levels in the blood were then measured at various time points post-injection. Unmodified exendin-4 and an equivalent volume of PBS were separately injected as a positive and negative control. We found that all conjugates significantly extended the glucose-lowering effect of exendin-4. The exendin-C-PEG_{10k} conjugate provided extended glucose reduction for up to 24 h ($P<0.05$; Figure 3e) whereas the larger exendin-C-PEG_{30k} conjugate reduced the blood glucose level for up to 48 h ($P<0.001$; Figure 3f). These results are in stark contrast with the temporally very limited activity of native exendin-4, which only lowers glucose levels for 4 h following s.c. injection (Figure 3d). Remarkably, a single s.c. injection of exendin-C-$PMPC_{28k}$ resulted in blood glucose reduction for up to 48 h ($P<0.05$; Figure 3e), and the larger exendin-C-$PMPC_{75k}$ lowered the blood glucose level for up to 72 h ($P<0.05$; Figure 3f). Clearly both PEG and a phosphorylated stealth polymer markedly extended the duration of the efficacy of exendin-4. Furthermore, a similar trend was observed for the mouse body weights, which decreased with the glucose levels (Figure S12), and is consistent with the weight-lowering benefit of exendin-4.[22] It is worth pointing out that these data demonstrate, for the first time, that attaching a zwitterionic polymer to the C terminus significantly improves the therapeutic efficacy of a peptide drug and is at least as good as the gold standard PEG.

In summary, we have reported a new and general method to engineer precisely defined protein/peptide–polymer therapeutics that are site-specific, stoichiometric, and formed in high yield by a combination of recombinant expression, enzyme-mediated ligation, and click chemistry. This modular approach is significant because 1) it can be used with various protein/peptide drugs, 2) it is suitable for the conjugation of structurally diverse polymers, 3) all steps involved in this method exhibit high yields and avoid the use of any organic solvent and catalyst, and 4) the synthesized protein/peptide–polymer conjugates display significantly improved pharmacokinetics and therapeutic effects. This conjugation method is likely to be useful in a manufacturing setting as it provides the desired compounds in high yield with low reaction times, and is experimentally robust in its tolerance to structurally diverse proteins and polymers. We believe that this method represents an important expansion of the methods currently available for polymer conjugation to proteins, with attributes that make it suitable for the clinical and commercial development of polymer conjugates that improve the delivery of protein/peptide therapeutics.

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[1] G. Walsh, *Nat. Biotechnol.* **2014**, *32*, 992–1000.
[2] B. Leader, Q. J. Baca, D. E. Golan, *Nat. Rev. Drug Discovery* **2008**, *7*, 21–39.
[3] V. H. L. Lee, *Peptide and protein drug delivery*, Mercel Dekker, New York, **1991**.
[4] M. Werle, A. Bernkop-Schnurch, *Amino Acids* **2006**, *30*, 351–367.
[5] A. K. Banga, *Parenteral controlled delivery and pharmacokinetics of therapeutic peptides and proteins. Therapeutic Peptides and Proteins*, CRC, Boca Raton, FL, **2005**, pp. 177–227.
[6] a) M. Hamidi, A. Azadi, P. Rafiei, *Drug Delivery* **2006**, *13*, 399–409; b) R. Bansal, E. Post, J. H. Proost, A. de Jager-Krikken, K. Poelstra, J. Prakash, *J. Controlled Release* **2011**, *154*, 233–240; c) R. Duncan, *Nat. Rev. Cancer* **2006**, *6*, 688–701; d) T. H. Kim, H. H. Jiang, S. M. Lim, Y. S. Youn, K. Y. Choi, S. Lee, X. Chen, Y. Byun, K. C. Lee, *Bioconjugate Chem.* **2012**, *23*, 2214–2220; e) M. Liu, P. Johansen, F. Zabel, J.-C. Leroux, M. A. Gauthier,

Nat. Commun. **2014**, *5*, 5526 – 5533; f) A. J. Keefe, S. Y. Jiang, *Nat. Chem.* **2012**, *4*, 59 – 63.
[7] a) R. M. Broyer, G. N. Grover, H. D. Maynard, *Chem. Commun.* **2011**, *47*, 2212 – 2226; b) V. Gaberc-Porekar, I. Zore, B. Podobnik, V. Menart, *Curr. Opin. Drug Discov. Devel.* **2008**, *11*, 242 – 250; c) B. Le Droumaguet, J. Nicolas, *Polym. Chem.* **2010**, *1*, 563 – 598; d) B. S. Sumerlin, *ACS Macro Lett.* **2012**, *1*, 141 – 145; e) M. A. Gauthier, H. Klok, *Chem. Commun.* **2008**, 2591 – 2611; f) J. D. Schulz, M. Patt, S. Basler, H. Kries, D. Hilvert, M. A. Gauthier, J.-C. Leroux, *Adv. Mater.* **2016**, *28*, 1455 – 1460.
[8] a) M. J. Roberts, M. D. Bentley, J. M. Harris, *Adv. Drug Delivery Rev.* **2002**, *54*, 459 – 476; b) Y. Qi, A. Chilkoti, *Polym. Chem.* **2014**, *5*, 266 – 276.
[9] A. Lewis, Y. Tang, S. Brocchini, J.-W. Choi, A. Godwin, *Bioconjugate Chem.* **2008**, *19*, 2144 – 2155.
[10] J. A. Johnson, Y. Y. Lu, J. A. Van Deventer, D. A. Tirrell, *Curr. Opin. Chem. Biol.* **2010**, *14*, 774 – 780.
[11] M. W. Popp, J. M. Antos, H. L. Ploegh, *Curr. Protoc. Protein Sci.* **2009**, *56*, 15.3.1 – 15.3.9.
[12] X. Ding, N. K. Saxena, S. Lin, N. A. Gupta, F. A. Anania, *Hepatology* **2006**, *43*, 173 – 181.
[13] J. Boekhorst, M. W. de Been, M. Kleerebezem, R. J. Siezen, *J. Bacteriol.* **2005**, *187*, 4928 – 4934.
[14] D. E. Meyer, A. Chilkoti, *Nat. Biotechnol.* **1999**, *14*, 1112 – 1115.
[15] H. Mao, S. A. Hart, A. Schink, B. A. Pollok, *J. Am. Chem. Soc.* **2004**, *126*, 2670 – 2671.
[16] a) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599; *Angew. Chem.* **2002**, *114*, 2708 – 2711; b) M. F. Debets, S. S. van Berkel, S. Schoffelen, F. P. J. T. Rutjes, J. C. M. van Hest, F. L. van Delft, *Chem. Commun.* **2010**, *46*, 97 – 99; c) L. M. Gaetke, C. K. Chow, *Toxicology* **2003**, *189*, 147 – 163.
[17] Y. S. Youn, D. H. Na, K. C. Lee, *J. Controlled Release* **2007**, *117*, 371 – 379.
[18] a) A. Bond, *Proc. (Bayl. Univ. Med. Cent.)* **2006**, *19*, 281 – 284; b) D. J. Drucker, *Diabetes* **1998**, *47*, 159 – 169; c) D. G. Parkes, K. F. Mace, M. E. Trautmann, *Expert Opin. Drug Delivery* **2013**, *8*, 219 – 244; d) P. A. Kothare, H. Linnebjerg, Y. Isaka, K. Uenaka, A. Yamamura, K. P. Yeo, A. Pena, C. H. Teng, K. Mace, M. Fineman, H. Shigeta, Y. Sakata, S. Irie, *J. Clin. Pharmacol.* **2008**, *48*, 1389 – 1399.
[19] R. Goke, H.-C. Fehmann, T. Linn, H. Schmidt, M. Krause, J. Eng, B. Goke, *J. Biol. Chem.* **1993**, *268*, 19650 – 19655.
[20] M. Amiram, K. M. Luginbuhl, X. H. Li, M. N. Feinglos, A. Chilkoti, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 2792 – 2797.
[21] M. S. Winzell, B. Ahren, *Diabetes* **2004**, *53*, S215 – S219.
[22] C. M. Mack, C. X. Moore, C. M. Jodka, S. Bhavsar, J. K. Wilson, J. A. Hoyt, J. L. Roan, J. L. Vu, K. D. Laugero, D. G. Parkes, A. A. Young, *Int. J. Obes.* **2006**, *30*, 1332 – 1340.

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