Supramolecular PEGylation of biopharmaceuticals

Supramolecular PEGylation of biopharmaceuticals

Matthew J. Webber[a,b,1,2], Eric A. Appel[a,1,3], Brittany Vinciguerra[c], Abel B. Cortinas[d], Lavanya S. Thapa[a,b], Siddharth Jhunjhunwala[a], Lyle Isaacs[c,4], Robert Langer[a,b,d,e,f,4], and Daniel G. Anderson[a,b,d,e,f,4]

[a]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; [b]Department of Anesthesiology, Boston Children's Hospital, Boston, MA 02112; [c]Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742; [d]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139; [e]Harvard–Massachusetts Institute of Technology Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139; and [f]Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Robert Langer, October 10, 2016 (sent for review July 15, 2016; reviewed by Jason A. Burdick and Justin Hanes)

The covalent modification of therapeutic biomolecules has been broadly explored, leading to a number of clinically approved modified protein drugs. These modifications are typically intended to address challenges arising in biopharmaceutical practice by promoting improved stability and shelf life of therapeutic proteins in formulation, or modifying pharmacokinetics in the body. Toward these objectives, covalent modification with poly(ethylene glycol) (PEG) has been a common direction. Here, a platform approach to biopharmaceutical modification is described that relies on noncovalent, supramolecular host–guest interactions to endow proteins with prosthetic functionality. Specifically, a series of cucurbit[7]uril (CB[7])–PEG conjugates are shown to substantially increase the stability of three distinct protein drugs in formulation. Leveraging the known and high-affinity interaction between CB[7] and an N-terminal aromatic residue on one specific protein drug, insulin, further results in altering of its pharmacological properties in vivo by extending activity in a manner dependent on molecular weight of the attached PEG chain. Supramolecular modification of therapeutic proteins affords a noncovalent route to modify its properties, improving protein stability and activity as a formulation excipient. Furthermore, this offers a modular approach to append functionality to biopharmaceuticals by noncovalent modification with other molecules or polymers, for applications in formulation or therapy.

supramolecular chemistry | protein engineering | drug delivery | protein formulation

The practice of medicine has been transformed by use of biopharmaceuticals, with many drugs coming to market in recent years in the form of peptides, proteins, and antibodies (1). These new classes of drugs have an array of attendant complications arising from poor chemical and/or structural stability that can lead to the active drug being converted into an inactive and/or potentially immunogenic form (2, 3). Extensive efforts have been devoted to the development of excipients for use in the formulation of proteins to confer improved stability (4). Excipients commonly used in the formulation of protein drugs include salts, sugars, amino acids, nonionic surfactants, chelators, antimicrobial preservatives, carrier proteins, and polymers (5). An alternate approach to promote stability or modify pharmacological activity of protein drugs is through direct chemical modification with a prosthetic functional group, commonly a synthetic polymer such as poly(ethylene glycol) (PEG) or a saturated alkyl segment (6–9). Specifically, PEGylation is known to increase protein solubility, limit access by proteolytic enzymes or opsonins, reduce glomerular filtration, and inhibit aggregation (10, 11). Covalently PEGylated biopharmaceuticals have been used clinically for a range of diseases, for example in conjunction with IFN treatment of hepatitis B, hepatitis C, and multiple sclerosis (12, 13). Direct covalent modification, although demonstrating efficacy in altering the stability and function of protein drugs, introduces complications from the need to isolate and purify the modified protein following labeling as well as the possibility that introduction of an exogenous moiety could lead to immunogenicity or a deleterious effect on protein function and signaling.

Supramolecular chemistry has broad potential application for biology and medicine by leveraging specific, directional, and reversible noncovalent molecular recognition motifs (14). Host–guest motifs, for example, typically comprise a discrete macrocyclic host with a cavity that is selective for complementary binding to certain guest ligands (15). The affinity of a number of hydrophobic small-molecule drugs with macrocyclic hosts has been leveraged to solubilize a wide variety of small-molecule pharmaceutical compounds (16, 17). One particular macrocycle, cucurbit[7]uril (CB[7]), is distinguished by extraordinarily strong binding affinities, including its participation among the strongest ever-reported receptor–ligand interactions (18). It has also been demonstrated to bind strongly ($K_{eq} \sim 10^6$ M^{-1}) to proteins with N-terminal aromatic amino acids (e.g., tryptophan and phenylalanine) using a combination of R-group inclusion and electrostatic interaction between the N-terminal ammonium and carbonyls on the CB[7] portal. Moreover, CB[7] may also bind midchain aromatic or cationic ammonium residues by either R-group inclusion or electrostatic interactions ($K_{eq} \sim 10^3$ to 10^4 M^{-1}) (19). It is important to note that, although supramolecular affinity for CB[7] with midchain amino acids is lower than that for N-terminal aromatics, it still represents significant affinity in the context of other commonly used host–guest supramolecular motifs; for example,

APPLIED PHYSICAL SCIENCES

Significance

Pharmaceutical practice has transitioned away from small-molecule drugs to the use of biomolecules (peptides, proteins, and antibodies). Where formulation of small molecules focused primarily on solubility, biopharmaceuticals introduced an array of complications due to their more complex secondary and tertiary structures, contributing to concerns surrounding aggregation and denaturation over time in formulation. Here, we outline an approach using noncovalent supramolecular affinity to endow biopharmaceuticals with a polymer known to inhibit protein aggregation and improve solubility. This method stands in contrast to similar approaches to covalently graft the same polymer onto the protein, instead offering a broadly useful and modular formulation excipient that can be combined with authentic unmodified protein drugs to extend shelf life.

Author contributions: M.J.W., E.A.A., and D.G.A. designed research; M.J.W., E.A.A., B.V., A.B.C., L.S.T., and S.J. performed research; B.V. and L.I. contributed new reagents/analytic tools; M.J.W. and E.A.A. analyzed data; and M.J.W., E.A.A., L.I., R.L., and D.G.A. wrote the paper.

Reviewers: J.A.B., University of Pennsylvania; and J.H., Johns Hopkins University School of Medicine.

Conflict of interest statement: R.L. and J.H. are co-founders of Kala Pharmaceuticals, Inc.

[1]M.J.W. and E.A.A. contributed equally to this work.

[2]Present address: Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN 46556.

[3]Present address: Department of Materials Science and Engineering, Stanford University, Stanford, CA 94304.

[4]To whom correspondence may be addressed. Email: lisaacs@umd.edu, rlanger@mit.edu, or dgander@mit.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1616639113/-/DCSupplemental.

host–guest binding involving cyclodextrin, one of the most commonly used supramolecular hosts in pharmaceutical practice, has a maximum observed affinity for any guest of ∼10^4 M^{-1} (20). Should even higher CB[7] binding affinities be desired than are possible with binding to native amino acids, prosthetic moieties could be added to proteins to achieve binding with K_{eq} upward of 10^{10} to 10^{15} M^{-1} (21–23).

Herein, we report on a CB[7] macrocyclic host modified with a single PEG chain for use in the supramolecular PEGylation of biopharmaceuticals via host–guest complexation between CB[7] and residues on the protein drug. This route for supramolecular PEGylation is demonstrated to enhance the stability of formulated protein drugs in vitro by preventing aggregation, and also provides longer-lasting pharmacological activity in vivo for a specific protein drug, insulin, containing a high-affinity N-terminal aromatic phenylalanine residue. This strategy could be used as platform excipient to endow biopharmaceuticals with the benefits of PEGylation without necessitating covalent modification of the drug itself, and could point to an even broader strategy to endow protein drugs with functional molecules or polymers without necessitating covalent attachment.

Results and Discussion

Although CB[7] exhibits exceptional binding affinities for a broad range of guest molecules (18, 21, 22, 24), it has been significantly more difficult to functionalize or conjugate than other classes of cyclic host molecules (25, 26). Three synthetic routes have recently been described to prepare functionalized CB[7] derivatives with synthetically useful chemical handles (27–30). Using the Isaacs group methods, an azido-functional CB[7] derivative (CB[7]–N_3) was synthesized to conjugate the macrocycle to PEG using copper-free "click" chemistry. PEG of three different molecular weights (5, 10, and 30 kDa), having a single terminal modification of dibenzocyclooctyne (DBCO), were reacted with CB[7]–N_3 to yield CB[7]–PEG consisting of a single PEG chain connected via a triazole linkage to the CB[7] macrocycle (Fig. 1). Matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) and NMR confirmed formation of the products (Figs. S1 and S2). Furthermore, no CB[7] starting material could be detected by electrospray ionization MS. Gel permeation chromatography (GPC) with UV/visible detection (Fig. 2 *A* and *B*) was performed to further verify the reaction between PEG–DBCO and CB[7]–N_3 and characterize the resulting CB[7]–PEG. The UV/visible spectrum upon GPC elution demonstrated a characteristic change in absorbance from reaction of the PEG–DBCO starting material to CB[7]–PEG by triazole formation, with a blue shift in λ_{max} from 290 to 248 nm. Moreover, the DBCO and triazole signatures appeared concurrent with elution of the polymeric backbone, as indicated by the refractive index trace. Additionally, MALDI-MS demonstrated a characteristic increase in molecular weight for CB[7]–PEG relative to the PEG–DBCO starting material (Fig. 2*C*) with a shift in the observed spectrum following reaction of the 30-kDa PEG–DBCO with CB[7]–N_3 that corresponded to a mass increase of ∼1,152 Da, consistent with addition of a single CB[7]–N_3 moiety. Other conjugates showed a similar increase relative to each PEG–DBCO starting material (Fig. S1). Taken together, these studies confirmed both facile and quantitative conversion of CB[7]–N_3 and PEG–DBCO starting materials to yield CB[7]–PEG conjugates of three distinct PEG lengths.

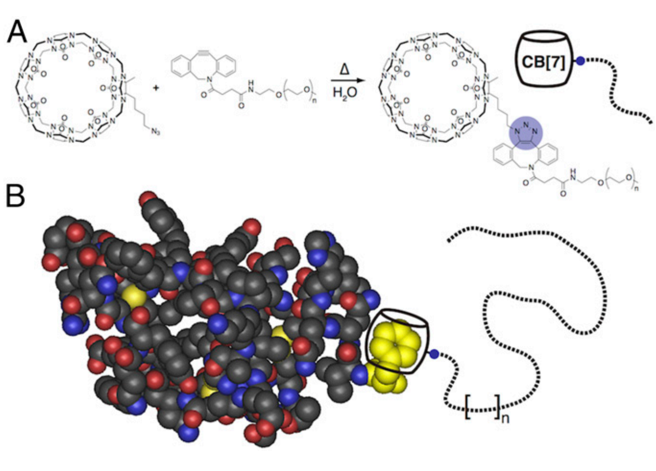

Fig. 1. Strategy for supramolecular PEGylation. (*A*) A copper-free "click" reaction between a cucurbit[7]uril (CB[7]) supramolecular host molecule bearing a single azide moiety (CB[7]–N_3) and a dibenzocyclooctyne-functional poly(ethylene glycol) polymer (PEG–DBCO) (M_n = 5, 10, or 30 kDa) yields CB[7]–PEG upon triazole formation (n.b., only one of two possible regioisomers for triazole formation is shown, highlighted in blue). (*B*) Cartoon depicting supramolecular PEGylation of the insulin protein through strong noncovalent binding of the CB[7] moiety to the N-terminal phenylalanine residue.

Insulin—used to treat diabetes and one of the most common biopharmaceuticals—has limited stability and is prone to the formation of immunogenic fibrillar aggregates in physiologic conditions (31, 32). Site-specific PEGylation of insulin has been achieved to control both its physical stability and pharmacological properties, and was shown to reduce aggregation, fibril formation, immunogenicity, and allergenicity, as well as increase serum half-life (33). Insulin also presents an N-terminal aromatic phenylalanine that has demonstrated high-affinity binding to CB[7] (34). Indeed, the known affinity for this N-terminal aromatic residue is expected to be 100- to 1,000-fold higher than that for any other residue on insulin, creating a strong preference for CB[7] binding specifically at this site. Therefore, insulin is an ideal candidate protein to assess supramolecular PEGylation using CB[7]–PEG with varying molecular weight PEG chains. To confirm binding of CB[7]–PEG to recombinant insulin, a competitive binding assay was performed using acridine orange (AO) (Fig. 2*D*). In this assay, the fluorescence emission spectrum of AO is collected in water. Because the fluorescence intensity of AO increases when complexed with CB[7] relative to its emission when free in water, reduction in fluorescence resulting from dye displacement is indicative of CB[7] binding to another ligand. As insulin is added, the CB[7]·AO complex dissociates due to the stronger binding affinity of the N-terminal phenylalanine residue to CB[7], leading to a reduction to the measured fluorescence intensity of AO. When fitting these data to a one-site competitive binding model, no significant difference in the binding constant toward insulin was observed in comparing unmodified CB[7] to CB[7]–PEG (e.g., CB[7], $K_D = 2.3 \pm 0.2$ μM, vs. CB[7]–PEG_{30k}, $K_D = 2.1 \pm 0.3$ μM). Interestingly, the presence of a pendant PEG chain of at least 30 kDa did not alter CB[7] binding to insulin, indicating no steric inhibition of CB[7] binding to the 5.8-kDa insulin protein. Furthermore, CB[7]–PEG binding to insulin did not result in changes to the secondary structure of the insulin protein, as measured by circular dichroism spectroscopy (Fig. S3).

To determine whether CB[7]–PEG binding stabilizes insulin and prevents its aggregation, assays were performed in physiologic salt concentrations using PBS buffer at pH 7.4 and 37 °C with continuous agitation to determine the kinetics of insulin aggregation in a solution of 1 mg/mL protein (Fig. 3*A*). Specifically, transmittance was measured at 540 nm, a wavelength at which insulin and CB[7]–PEG have negligible absorbance, to detect the formation of aggregates that scatter light formed in solution over time. Defining aggregation time (t_A) as the time upon which a 10% reduction in transmittance is observed, recombinant insulin aggregated following 13.6 ± 0.2 h of agitation while insulin formulated with CB[7] aggregated following 14.2 ± 0.4 h. Meanwhile, insulin formulated with each molecular weight of CB[7]–PEG did not aggregate during the 100-h kinetic study. This finding is important, as it shows that CB[7] binding to insulin alone does not contribute to improved formulation stability. As a control, the addition of PEG polymers of comparable molecular weights alone or in combination with unmodified CB[7] was not found to prolong insulin stability beyond that observed for insulin alone (Fig. S4), highlighting specifically the importance of supramolecular PEGylation using CB[7] conjugated to PEG in enhancing the stability of recombinant insulin in formulation. After the initial

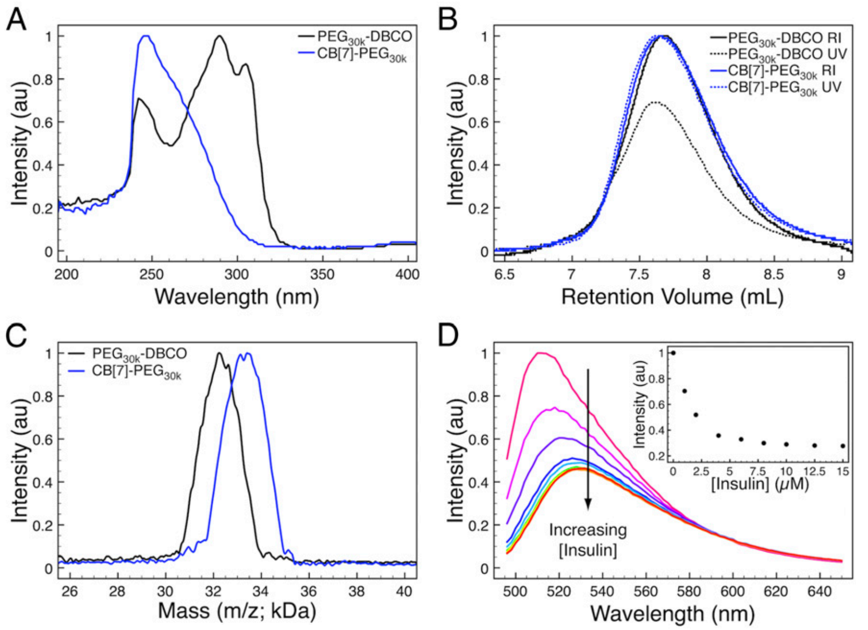

Fig. 2. Characterization of CB[7]–PEG conjugates. (*A*) Gel permeation chromatography (GPC) showing UV/visible absorbance of PEG_{30k}-DBCO and CB[7]–PEG_{30k} indicates a change in the λ_{max} of the DBCO moiety from 290 to 248 nm upon conjugation of the CB[7]–N_3 and corresponding formation of a triazole. (*B*) Overlay of refractive index and absorbance (triazole λ_{max} = 245 nm) traces from GPC of PEG_{30k}-DBCO and CB[7]–PEG_{30k} demonstrates coelution of the triazole signature with the polymeric species. (*C*) MALDI-MS characterization confirms an increase in mass of the PEG_{30k} species corresponding to conjugation of the CB [7] moiety. Peaks: 32,262 Da (starting material, black) and 33,414 Da (conjugate, blue). ΔMass = 1,152 Da. (*D*) Acridine orange (AO) competitive binding assay with recombinant insulin, showing a decay in AO fluorescence upon addition of insulin, indicates no significant alteration of the CB[7]–insulin binding occurs following PEG conjugation (CB[7], $K_D = 2.3 \pm 0.2$ μM; CB[7]–PEG_{5k}, $K_D = 1.4 \pm 0.1$ μM; CB[7]–PEG_{10k}, $K_D = 1.3 \pm 0.1$ μM; CB[7]–PEG_{30k}, $K_D = 2.1 \pm 0.3$ μM), using AO (8 μM), CB[7] or CB[7]–PEG (6 μM), and insulin (varied from 0 to 15 μM), with binding studies performed in water. The intensity at 510 nm was used in fitting the decay of signal, shown in the *Inset*. Representative data for CB[7]–PEG_{30k} binding to insulin is shown for the AO assay and *Inset*.

100-h kinetic study, transmittance of the insulin samples formulated with CB[7]–PEG was measured once daily. No aggregation was observed at the 100-d endpoint of these studies. Thus, formulation of insulin with each of the three CB[7]–PEG conjugates extended stability in solution, from ∼14 h to over 100 d. Subsequently, in vitro activity was measured for all insulin formulations after 100 d of aging through dose–response studies of AKT phosphorylation (Fig. 3*B*). When insulin had been formulated with each of the CB[7]–PEG conjugates and aged for 100 d under physiologic conditions with agitation, the $LogEC_{50}$ was comparable to that for freshly dissolved insulin. However, a two-log reduction in activity was observed for insulin that had been aged for 100 d alone or in formulation with CB[7]. Taken together with data for insulin aggregation, formulation with CB[7]–PEG was found to preserve both the stability and activity of insulin for up to 100 d in stressed conditions, whereas insulin alone as well as that formulated with unmodified CB[7] aggregated within ∼14 h and was significantly less active.

Glucagon and an antibody for human CD20—a variant of which is used clinically as Rituximab—were also evaluated to determine whether CB[7]–PEG exhibits a stabilizing effect that extends to formulation with other proteins. Despite an absence of high-affinity N-terminal aromatic amino acid guests that exist on insulin, CB[7]–PEG was still expected to bind with significant affinity to midchain residues on these proteins. Importantly, at concentrations used typically for formulation of biopharmaceuticals (i.e., on the order of 1 mg/mL), significant binding would still be expected even at these lower regimes for supramolecular affinity. Anti-CD20 was aged under agitated conditions alone or in formulation with the various CB[7]–PEG conjugates for 24 h in PBS at room temperature. Following this time, its function was measured through reactivity with a human B-cell lymphoma cell line known to display a high density of CD20 on its surface (Fig. 4*A*). In the case where agitation was performed in the presence of CB[7]–PEG, the antibody retained its activity in binding to these cells, evidenced by fluorescence intensity values from flow cytometry comparable to those for the fresh antibody. However, when the antibody was agitated in formulation alone or with unmodified CB[7], a significant reduction in activity was observed with a two-log decrease in the fluorescence signal, approximately equivalent to the signal produced from these cells when no antibody had been added. In the case of glucagon, the protein typically remains soluble for less than 1 h before undergoing aggregation and precipitation, which can result in the formation of cytotoxic amyloidogenic fibrils (35). Poor glucagon stability has also proved limiting to its use as a component in bihormonal diabetes therapy (36). Here, when glucagon was formulated with CB[7]–PEG, it remained soluble for at least 24 h in solution, whereas glucagon alone precipitated from

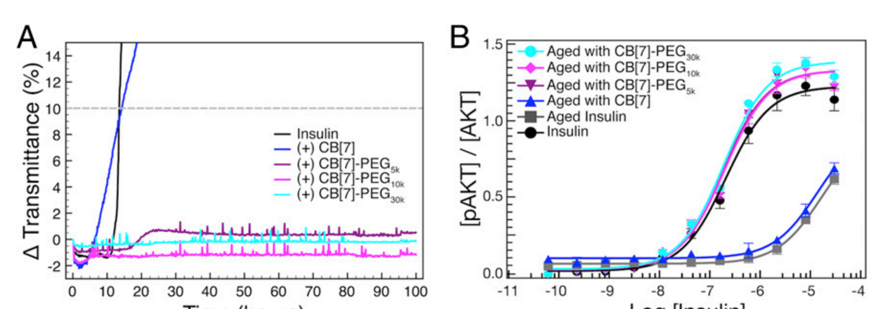

Fig. 3. In vitro insulin stability and activity. (*A*) Kinetic profiling of the aggregation of various insulin formulations (through measurements of solution transmittance) at pH 7.4, 37 °C, in physiological buffer with continuous agitation over the course of 100 h, demonstrating insulin formulation with CB[7]–PEG to resist aggregation over the period assayed. (Note: monitoring was continued daily for 100 d, with no change in transmittance observed for insulin formulated with CB[7]–PEG.) Data shown are the average transmittance trace for $n = 4$ samples per group. (*B*) Following 100 d of agitated aging, insulin formulated with CB[7]–PEG conjugates retains its activity when assaying for phosphorylation of Ser^{473} on AKT, whereas insulin aged alone or with CB[7] show limited activity (potency: aged insulin, $LogEC_{50} = -4.8 \pm 0.06$; CB[7], $LogEC_{50} = -4.9 \pm 0.07$; CB[7]–$PEG_{5k}$, $LogEC_{50} = -6.8 \pm 0.04$; CB[7]–$PEG_{10k}$, $LogEC_{50} = -6.7 \pm 0.04$; CB[7]–$PEG_{30k}$, $LogEC_{50} = -6.7 \pm 0.04$; fresh insulin, $LogEC_{50} = -6.8 \pm 0.04$). Data shown are the average of $n = 3$ experimental points, each prepared from a pool of four samples that had been aged for 100 d, with error bars showing the SD between the three experimental replicates. Statistical significance determined by an ANOVA with Bonferroni multiple-comparison post hoc test for the fit statistics of $LogEC_{50}$ with n = fit degrees of freedom, yielded the following significance: $P < 0.0001$ for fresh insulin, CB[7]–PEG_{5k}, CB[7]–PEG_{10k}, and CB[7]–PEG_{30k} vs. CB[7] and aged insulin.

solution in under 1 h (Fig. 4*B*). In addition, glucagon remained in its native and active α-helical conformation for at least 24 h when formulated with CB[7]–PEG (Fig. 4*C*), confirming no amyloid formation during this time.

Taken together, formulation with CB[7]–PEG conjugates provides a method to preserve the stability of protein drugs when used as a formulation excipient. Specifically, this method was found to preserve the stability and function of three very different protein drugs—insulin, glucagon, and an antibody—indiscriminate of their primary sequence, molecular weight, or secondary structure. Even in the case where the protein did not contain an N-terminal aromatic residue, supramolecular interactions with the proteins in combination with standard formulation concentrations were sufficient to impart PEGylation benefits on these therapeutic biomolecules. As such, CB[7]–PEG is proposed as a versatile and modular platform excipient to facilitate increased stability and shelf life of biopharmaceuticals, thereby limiting the formation of inactive and possibly immunogenic aggregates in formulation.

Insulin has additional interest in that it presents a N-terminal aromatic guest that binds with an affinity upward of 1,000-fold higher than proteins lacking such a feature (34). Affinities this high might thus facilitate complex formation for certain in vivo scenarios where a weaker affinity interaction would disassemble due to rapid dilution or competition from other proteins. Specifically, a s.c. depot that arises following routine injection of insulin might afford an environment favorable to short-term interaction of the N-terminal phenylalanine on the protein with CB[7] through limiting dilution and competition. As such, the effect of CB[7]–PEG on the pharmacological properties of insulin in vivo was probed further. A mouse model of insulin-deficient diabetes, prepared using streptozotocin to induce pancreatic beta-cell death (37), was used to evaluate the function of CB[7]–PEG delivered with insulin (Fig. 5). Following the onset of diabetes, mice were fasted overnight to ensure an average starting blood glucose level of ∼450 mg/dL. Recombinant insulin was then administered s.c. at a dose of 1 IU/kg in a saline vehicle. Alternatively, an identical dose of insulin was administered along with CB[7], CB[7]–PEG$_{5k}$, CB[7]–PEG$_{10k}$, or CB[7]–PEG$_{30k}$. All insulin formulations reduced average blood glucose to a normoglycemic level (<200 mg/dL for a mouse) following a single s.c. administration. The slope of this initial drop in blood glucose appears independent of formulation and likely arises from the reported dose-independent sensitivity of the streptozotocin (STZ) mouse model for administered insulin at short time intervals following administration (38). Animals that received either recombinant insulin or insulin formulated with CB[7] became hyperglycemic again ∼2 h following administration. However, animals that received insulin formulated with CB[7]–PEG$_{5k}$ and CB[7]–PEG$_{10k}$ remained normoglycemic for ∼3 and 5 h, respectively, following administration, whereas insulin formulated with CB[7]–PEG$_{30k}$ preserved normoglycemia until the 6-h endpoint of the study. At this 6-h endpoint, the average blood glucose level of mice in this group was 129 mg/dL, well within a normoglycemic range. Thus, the duration of activity for recombinant insulin was directly dependent on molecular weight of the PEG chain attached to CB[7]. Pharmacological function of the authentic recombinant insulin protein can therefore be controlled by noncovalent modification, enabling the duration of therapy to be tuned through modular selection of PEG chain length without a need for direct modification to the therapeutic protein.

The mechanism by which CB[7]–PEG extends the activity of insulin in vivo is most likely attributable to the known depot effect for insulin administration (39). Insulin administered s.c., which is the most common route for patient administration and which was performed here, must reach vascular circulation before eliciting its function. s.c. administration of compounds <1 kDa in molecular weight are typically preferentially absorbed directly via capillary circulation, whereas larger compounds (including insulin) primarily leverage interstitial fluid flux of lymphatic circulation to reach the vasculature, resulting in delayed systemic bioavailability (40–42). Moreover, it is known that molecular weight (and by extension, viscosity) affects the rate at which compounds can traffic to the lymphatic circulation (43). Therefore, supramolecular PEGylation of insulin using PEG chains of various molecular weights likely contributes to delayed and controlled uptake, creating a sustained source of insulin in the s.c. space by increasing the effective molecular weight of the complex as a result of CB[7]–PEG binding. PEGylation is also well known to provide a steric protective effect on proteins in limiting access by proteolytic enzymes (10–13, 44), a role that may also be filled by supramolecular PEGylation in this case. CB[7] binding has also been demonstrated to protect proteins from protease activity (45). It has previously been speculated that binding of CB[7] to the N-terminal phenylalanine is not likely to occur to any significant extent in circulation in vivo because normal insulin concentration in serum is well below the K_D for binding of CB[7] to insulin; additionally, a high concentration of serum proteins that bind more weakly to CB[7] introduce competition (34). Although there may be a multifaceted effect of the CB[7]–PEG on insulin properties in vivo, the underlying mechanism for the findings here is likely to favor a s.c. depot effect, whereby the CB[7]–PEG conjugates slow the absorption of insulin into circulation and/or protect insulin from proteolysis within the artificially high, and thus preferential binding, environment of the depot. As the effective concentration within the depot would be significantly higher than that

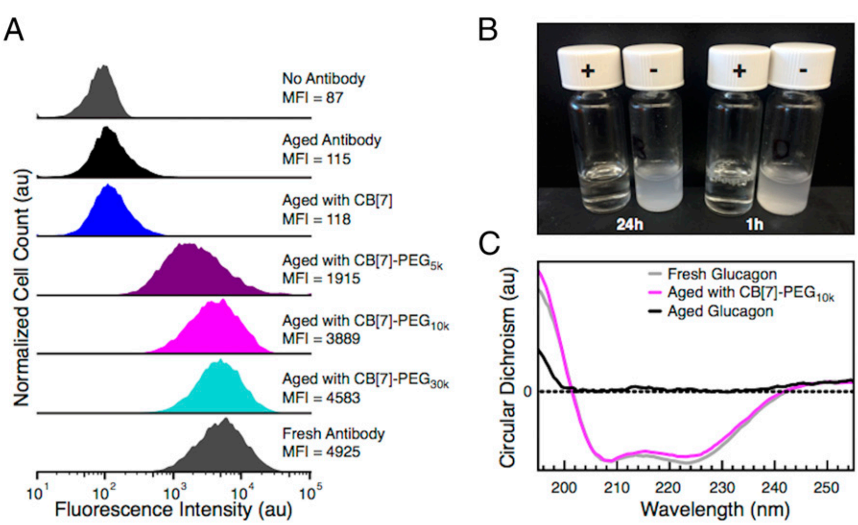

Fig. 4. Stability enhancement with other biopharmaceuticals. (*A*) Anti-CD20 antibody, aged in formulation with CB[7] or CB[7]–PEG, was evaluated for reactivity against CD20$^+$ Raji cells, analyzed by flow cytometry after incubation with cells and subsequent labeling with a fluorescent anti-IgG secondary antibody. Median fluorescent intensity (MFI) is shown for each group. Antibody reactivity of Raji cells is preserved only in the case where antibody is aged in the presence of CB[7]–PEG. (*B*) Glucagon, when formulated with all CB[7]–PEG conjugates, remains soluble for at least 24 h, whereas it readily precipitates from solution when dissolved alone. Shown is an illustrative example of this effect following formulation with CB[7]–PEG$_{10k}$ (+) compared with glucagon alone in solution (–). (*C*) Near-UV circular dichroism demonstrates glucagon aged for 24 h in the presence of CB[7]–PEG$_{10k}$ remains in an active α-helix conformation, whereas there is no signal attributable to glucagon in the aged samples. For circular dichroism studies, all samples were measured at identical protein concentrations using the same 100-μm-pathlength cuvette.

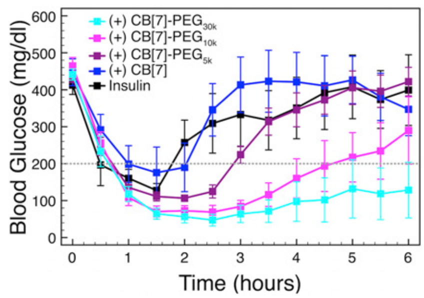

Fig. 5. In vivo modulation of insulin properties. In vivo assessment of blood glucose levels in STZ diabetic mice following administration of recombinant insulin (1 IU/kg) injected alone or formulated with CB[7] or CB[7]–PEG conjugates. Insulin was injected at $t = 0$, and blood glucose was monitored for 6 h following insulin administration ($n = 5$ mice/group). The dotted gray line shows the standard criteria for normoglycemic in this strain of mice (<200 mg/dL). Insulin administered in formulation with CB[7]–PEG conjugates demonstrated extended activity that was a function of molecular weight of the PEG chain. A one-way ANOVA with Bonferroni multiple-comparison post hoc test was performed at each hour, yielding significance as follows: at 2 h, $P < 0.05$ for CB[7]–PEG_{5k} and CB[7]–PEG_{10k} and CB[7]–PEG_{30k} vs. insulin. At 3 h, $P < 0.05$ for CB[7]–PEG_{10k} and CB[7]–PEG_{30k} vs. insulin and CB[7]. At 4 h and 5 h, $P < 0.05$ for CB[7]–PEG_{10k} and CB[7]–PEG_{30k} vs. insulin and CB[7] and CB[7]–PEG_{5k}. At 6 h, $P < 0.05$ for CB[7]–PEG_{30k} vs. insulin and CB[7] and CB[7]–PEG_{5k} and CB[7]–PEG_{10k}.

following uptake and dilution in the circulation, a depot would thus favor existence of the bound species.

Conclusions

In biopharmaceutical practice, assuring stability and modulating pharmacokinetics are two primary areas of importance; covalent PEGylation has been among the more successful strategies evaluated in this regard. Here, we have demonstrated supramolecular PEGylation of therapeutic proteins using CB[7]–PEG conjugates, outlining a versatile strategy to facilitate improved protein stability and limit aggregation for biopharmaceutical formulations. Contrary to conventional PEGylation of proteins, this approach uses interactions between a macrocyclic CB[7] host and amino acid side chains on the protein to endow proteins with PEG functionality noncovalently. Moreover, this excipient approach may have significant benefit over direct covalent PEGylation, namely, that the authentic therapeutic entity remains unmodified. This could have benefits in reducing the risk of immunogenicity for a supramolecular conjugate in comparison with a covalently modified one, as the PEG chain would be only transiently associated with the protein. Also, when used in the context of a formulation excipient, it is possible that supramolecular PEGylation would simplify regulatory approval of a previously approved biopharmaceutical in comparison with that for covalent PEGylation of the same protein. In cases where therapeutic proteins contain N-terminal aromatic residues, such as is the case for insulin, the higher-affinity interactions may also make possible prolongation of a therapeutic effect in the body through complex formation. This approach to supramolecular PEGylation thus warrants further exploration as a platform of "smart" excipients for formulation and shelf-life extension of virtually any biopharmaceutical, with additional function possible for proteins that contain N-terminal aromatic amino acids. Furthermore, the general strategy of endowing proteins with prosthetic functionality using CB[7] binding could extend to use with other polymers or functional groups beyond the PEG demonstrated here.

Methods

Synthesis of CB[7]–PEG Conjugates. Two synthetic routes have recently been described to prepare monofunctionalized CB[7] derivatives with synthetically useful handles (27–29). CB[7]–N_3 was synthesized according to the methods developed and reported previously by the laboratory of L.I. (27). This compound was then used to prepare supramolecular PEG conjugates using copper-free "click" chemistry. DBCO-functional PEG (PEG–DBCO) with molecular weights of 5, 10, and 30 kDa were purchased from Click Chemistry Tools, whereas monofunctional CB[7]–N_3 was synthesized as described previously (27). Commercially sourced PEG–DBCO derivatives (100 mg, 1.05 eq) were mixed with CB[7]–N_3 (1 eq) in H_2O/DMSO [4 mL, 1:1 (vol/vol)] and stirred at 60 °C for 24 h. The reaction mixture was lyophilized, and the product was characterized by MALDI-MS (Fig. S1) and ^{1}H-NMR (400 MHz; Fig. S2). GPC was performed in water on a glucose-modified divinylbenzene column using a Malvern Viscotek system with both refractive index and UV-visible detectors at a 1.0 mL/min flow rate. Samples were filtered through 0.2-μm PVDF filters before injection onto the column.

AO Competitive Binding Assay. For these studies, unmodified CB[7] was purchased from Strem Chemicals, recombinant human insulin was purchased from Life Technologies, and AO was purchased from Sigma-Aldrich. Binding of CB[7]–PEG conjugates to insulin was assessed using the AO dye displacement assay, in accordance with a previously described protocol (34). Briefly, 6 μM of either unmodified CB[7] or the CB[7]–PEG conjugates and 8 μM AO were combined with recombinant insulin (0, 1, 2, 4, 6, 8, 10, 12.5, 15 μM) in H_2O. Samples were incubated overnight in light-free conditions, and fluorescent spectra were collected on an Infinite M1000 plate reader (Tecan Group), exciting at 485 nm and collecting the resulting fluorescent spectra from 495 to 650 nm. The decay in the peak of AO fluorescent signal was fit to a one-site competitive binding model (GraphPad Prism, version 6.0), using the CB[7]·AO equilibrium constant reported previously ($K_{eq} = 2 \times 10^5\ M^{-1}$) (46), to determine binding constants of unmodified CB[7] and CB[7]–PEG to recombinant insulin.

Circular Dichroism. Recombinant human insulin was dissolved at 0.25 mg/mL in H_2O in combination with 1 eq of CB[7]–PEG, and samples were left to equilibrate for 3 h at room temperature. For glucagon studies, samples were evaluated immediately following the 24-h aggregation study without dilution. Near-UV circular dichroism spectroscopy was performed with a Jasco J-1500 high-performance circular dichroism spectrometer over a wavelength range of 190–250 nm using a 0.1-cm-pathlength cell.

Insulin Aggregation Assay. Recombinant human insulin was dissolved at 4 mg/mL in 2 mM HCl with 150 mM NaCl. Insulin was diluted to a final concentration of 1 mg/mL in PBS in combination with 1 eq of unmodified CB[7] or CB[7]–PEG conjugates. Samples were plated at 150 μL per well ($n = 4$/group) in a clear 96-well plate (Thermo Scientific Nunc) and sealed with optically clear and thermally stable seal (VWR). The plate was immediately placed into an Infinite M1000 plate reader (Tecan Group) and shaken continuously at 37 °C. Absorbance readings at 540 nm were collected every 6 min for 100 h, and absorbance values were subsequently converted to transmittance. Controls were also performed under identical conditions except with the addition of PEG with molecular weight of 10 or 30 kDa, without unconjugated CB[7]. The aggregation of insulin leads to light scattering, which results in reduction of sample transmittance. The time for aggregation (t_A) was defined as a >10% reduction in transmittance from the initial transmittance. Following the 100-h kinetic study, the plate was maintained under continuous agitation at 37 °C, and absorbance at 540 nm was monitored daily to approximate t_A for the CB[7]–PEG conjugates. At 100 d, with no indication of a change in absorbance for insulin samples formulated with CB[7]–PEG, the aggregation study was terminated, and insulin was assessed for activity using a cell-based assay.

In Vitro Insulin Activity. C2C12 cells were purchased from the American Type Culture Collection (ATCC) and confirmed free of mycoplasma contamination before use. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing L-glutamine, 4.5 g/L D-glucose, and 110 mg/L sodium pyruvate, and supplemented with 10% FBS and 1% penicillin–streptomycin. Incubations occurred in a 5% CO_2/water-saturated incubator at 37 °C. Cells were seeded in 96-well plates at a density of 5,000 cells per well. Twenty-four hours after plating, the cells were washed twice with 200 μL of DMEM containing L-glutamine, 4.5 g/L glucose, and 110 mg/L sodium pyruvate, and starved for 2 h at 37 °C in the same serum-free conditions. After 2 h, the media was removed and cells were stimulated with 100 μL of insulin samples of various concentrations for 30 min at 37 °C. After 30 min, cells were washed twice with 100 μL of cold Tris-buffered saline (1×), followed by lysing the cells for 10 min with 100 μL of cold Lysis Buffer (Perkin-Elmer). Levels of phosphorylated AKT 1/2/3 (Ser473) and total AKT 1 were determined from cell lysates using the AlphaLISA SureFire ULTRA kits (Perkin-Elmer) according to the manufacturer's instructions. Intrawell normalized data of phosphorylated AKT 1/2/3 (Ser473) and total AKT 1 were analyzed using GraphPad Prism 6.0, with dose–response curves fitted to a variable slope (three-parameter) stimulation model to determine the EC_{50} of each insulin sample.

Antibody Stability Assessment. Mouse anti-human CD20 IgG (Biolegend) was dissolved at 0.5 mg/mL in PBS alone, or with 2 eq of CB[7] or CB[7]–PEG conjugates

APPLIED PHYSICAL SCIENCES

for a total volume of 200 μL into 2-mL glass vials. Into each vial, five 1-mm polystyrene beads (Sigma) were added. The samples were subject to vortex agitation continuously for 24 h to induce aggregation. As a CD20-positive cell source, human Raji B-cell lymphoma cells (ATCC) were grown in RPMI 1640 media supplemented with 10% FBS and passaged four times before experiments. For each antibody sample, 1 million cells were mixed in 100 μL of various antibody formulations, diluted to a concentration of 1 μg/mL based on a dilution for this antibody determined in separate controls to be an appropriate dilution below saturation for these specific cells. Cells were incubated with anti-CD20 antibody (100 μL at 1 μg/mL) for 25 min, following which they were washed, combined with a secondary goat anti-mouse IgG labeled with Alexa Fluor 555 (1:500 dilution; Biolegend), and incubated for 25 min. Following additional washes, cells were suspended in 500 μL of buffer and analyzed on an LSRFortessa (BD Biosciences) flow cytometer. Data were analyzed by FlowJo, version 10.1.

Glucagon Stability Assessment. Human recombinant glucagon (Sigma) was dissolved at 4 mg/mL in PBS containing 1 mM HCl. This was mixed 1:1 with 1 eq of CB[7]–PEG in PBS, or in PBS alone, for a final glucagon concentration of 2 mg/mL. Vials were left to age at room temperature for various times. Circular dichroism was performed on samples without dilution, as well as on freshly dissolved glucagon, by using a 100-μm-pathlength cuvette.

In Vivo Insulin Performance. Male C57BL/6J, aged 8 wk, were purchased from The Jackson Laboratory. Upon acclimation for 1 wk in the animal facility, mice were fasted for 4 h before i.p. injection of 150 mg/kg STZ (Sigma). STZ for injection was dissolved at a concentration of 22.5 mg/mL in 2.94% (wt/vol) in pH 4.5 sodium citrate buffer immediately before injection. Mice were allowed to eat ad libitum, and glucose levels were monitored by peripheral tail vein bleeds using a portable glucose meter (Accu-Chek Aviva; Roche) daily until unfasted glucose levels >400 mg/dL. STZ-induced diabetic mice were fasted overnight before assessing insulin performance. Mice were bled at the beginning of the study, and any mouse with a fasting blood glucose level below 300 mg/dL was triaged from the study. Mice were then randomized and injected s.c. with insulin dosed at 1 IU/kg (34.7 μg/kg) either alone or formulated with 5 molar equivalents of unmodified CB[7] or CB[7]–PEG conjugates. In all cases, insulin with and without CB[7] or CB[7]–PEG was injected in a 200-μL saline vehicle. Blood glucose readings were collected every 30 min using a handheld glucose meter for 6 h following insulin injection. These studies were approved by the Massachusetts Institute of Technology Animal Care and Use Committee.

ACKNOWLEDGMENTS. We acknowledge technical support from Koch Institute Swanson Biotechnology Center and are specifically grateful for use of the Biopolymers and Proteomics Core and the Flow Cytometry Core. This work was supported by the Leona M. and Harry B. Helmsley Charitable Trust (Award 2014PG-T1D002), as well as a generous gift from the Tayebati Family Foundation. L.I. thanks the National Science Foundation (Grant CHE-1404911) for financial support. M.J.W. acknowledges support from the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases) through Ruth L. Kirschstein National Research Service Award F32DK101335. E.A.A. acknowledges support from a Wellcome Trust–Massachusetts Institute of Technology postdoctoral fellowship and a Margaret A. Cunningham award. B.V. acknowledges financial support from the Millard and Lee Alexander Fellowship (University of Maryland).

1. Aggarwal RS (2014) What's fueling the biotech engine—2012 to 2013. *Nat Biotechnol* 32(1):32–39.
2. Shire SJ, Shahrokh Z, Liu J (2004) Challenges in the development of high protein concentration formulations. *J Pharm Sci* 93(6):1390–1402.
3. Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W (2004) Structure-immunogenicity relationships of therapeutic proteins. *Pharm Res* 21(6):897–903.
4. Wang W (1999) Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm* 185(2):129–188.
5. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB (2011) Protein-excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development. *Adv Drug Deliv Rev* 63(13):1118–1159.
6. Keefe AJ, Jiang S (2011) Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. *Nat Chem* 4(1):59–63.
7. Duncan R (2006) Polymer conjugates as anticancer nanomedicines. *Nat Rev Cancer* 6(9):688–701.
8. Kurtzhals P, et al. (1995) Albumin binding of insulins acylated with fatty acids: Characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. *Biochem J* 312(Pt 3):725–731.
9. Alconcel SNS, Baas AS, Maynard HD (2011) FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polym Chem-Uk* 2(7):1442–1448.
10. Roberts MJ, Bentley MD, Harris JM (2002) Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev* 54(4):459–476.
11. Harris JM, Chess RB (2003) Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2(3):214–221.
12. Jevsevar S, Kunstelj M, Porekar VG (2010) PEGylation of therapeutic proteins. *Biotechnol J* 5(1):113–128.
13. Veronese FM, Pasut G (2005) PEGylation, successful approach to drug delivery. *Drug Discov Today* 10(21):1451–1458.
14. Webber MJ, Appel EA, Meijer EW, Langer R (2016) Supramolecular biomaterials. *Nat Mater* 15(1):13–26.
15. Schneider HJ (1991) Mechanisms of molecular recognition—investigations of organic host guest complexes. *Angew Chem Int Ed Engl* 30(11):1417–1436.
16. Davis ME, Brewster ME (2004) Cyclodextrin-based pharmaceutics: Past, present and future. *Nat Rev Drug Discov* 3(12):1023–1035.
17. Uekama K, Otagiri M (1987) Cyclodextrins in drug carrier systems. *Crit Rev Ther Drug Carrier Syst* 3(1):1–40.
18. Cao L, et al. (2014) Cucurbit[7]uril·guest pair with an attomolar dissociation constant. *Angew Chem Int Ed Engl* 53(4):988–993.
19. Urbach AR, Ramalingam V (2011) Molecular recognition of amino acids, peptides, and proteins by cucurbit[n]uril receptors. *Isr J Chem* 51(5-6):664–678.
20. Ma X, Zhao Y (2015) Biomedical applications of supramolecular systems based on host-guest interactions. *Chem Rev* 115(15):7794–7839.
21. Rekharsky MV, et al. (2007) A synthetic host-guest system achieves avidin-biotin affinity by overcoming enthalpy-entropy compensation. *Proc Natl Acad Sci USA* 104(52): 20737–20742.
22. Moghaddam S, et al. (2011) New ultrahigh affinity host-guest complexes of cucurbit[7]uril with bicyclo[2.2.2]octane and adamantane guests: Thermodynamic analysis and evaluation of M2 affinity calculations. *J Am Chem Soc* 133(10):3570–3581.
23. Lee DW, et al. (2011) Supramolecular fishing for plasma membrane proteins using an ultrastable synthetic host-guest binding pair. *Nat Chem* 3(2):154–159.
24. Liu S, et al. (2005) The cucurbit[n]uril family: Prime components for self-sorting systems. *J Am Chem Soc* 127(45):15959–15967.
25. Kim K, et al. (2007) Functionalized cucurbiturils and their applications. *Chem Soc Rev* 36(2):267–279.
26. Lee JW, Samal S, Selvapalam N, Kim HJ, Kim K (2003) Cucurbituril homologues and derivatives: New opportunities in supramolecular chemistry. *Acc Chem Res* 36(8):621–630.
27. Vinciguerra B, et al. (2012) Synthesis and self-assembly processes of monofunctionalized cucurbit[7]uril. *J Am Chem Soc* 134(31):13133–13140.
28. Ahn Y, Jang Y, Selvapalam N, Yun G, Kim K (2013) Supramolecular Velcro for reversible underwater adhesion. *Angew Chem Int Ed Engl* 52(11):3140–3144.
29. Cao L, Hettiarachchi G, Briken V, Isaacs L (2013) Cucurbit[7]uril containers for targeted delivery of oxaliplatin to cancer cells. *Angew Chem Int Ed Engl* 52(46):12033–12037.
30. Ayhan MM, et al. (2015) Comprehensive synthesis of monohydroxy-cucurbit[n]urils (n = 5, 6, 7, 8): High purity and high conversions. *J Am Chem Soc* 137(32):10238–10245.
31. Patterson CC, Dahlquist GG, Gyürüs E, Green A, Soltész G; EURODIAB Study Group (2009) Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: A multicentre prospective registration study. *Lancet* 373(9680):2027–2033.
32. Sluzky V, Tamada JA, Klibanov AM, Langer R (1991) Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. *Proc Natl Acad Sci USA* 88(21):9377–9381.
33. Hinds KD, Kim SW (2002) Effects of PEG conjugation on insulin properties. *Adv Drug Deliv Rev* 54(4):505–530.
34. Chinai JM, et al. (2011) Molecular recognition of insulin by a synthetic receptor. *J Am Chem Soc* 133(23):8810–8813.
35. Onoue S, et al. (2004) Mishandling of the therapeutic peptide glucagon generates cytotoxic amyloidogenic fibrils. *Pharm Res* 21(7):1274–1283.
36. Castle JR, et al. (2010) Novel use of glucagon in a closed-loop system for prevention of hypoglycemia in type 1 diabetes. *Diabetes Care* 33(6):1282–1287.
37. Like AA, Rossini AA (1976) Streptozotocin-induced pancreatic insulitis: New model of diabetes mellitus. *Science* 193(4251):415–417.
38. Chou DH, et al. (2015) Glucose-responsive insulin activity by covalent modification with aliphatic phenylboronic acid conjugates. *Proc Natl Acad Sci USA* 112(8):2401–2406.
39. Jockel JP, Roebrock P, Shergold OA (2013) Insulin depot formation in subcutaneous tissue. *J Diabetes Sci Technol* 7(1):227–237.
40. McLennan DN, Porter CJ, Charman SA (2005) Subcutaneous drug delivery and the role of the lymphatics. *Drug Discov Today Technol* 2(1):89–96.
41. Swartz MA (2001) The physiology of the lymphatic system. *Adv Drug Deliv Rev* 50(1-2):3–20.
42. Chertok B, Webber MJ, Succi MD, Langer R (2013) Drug delivery interfaces in the 21st century: From science fiction ideas to viable technologies. *Mol Pharm* 10(10):3531–3543.
43. Supersaxo A, Hein WR, Steffen H (1990) Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. *Pharm Res* 7(2):167–169.
44. Veronese FM (2001) Peptide and protein PEGylation: A review of problems and solutions. *Biomaterials* 22(5):405–417.
45. Logsdon LA, Urbach AR (2013) Sequence-specific inhibition of a nonspecific protease. *J Am Chem Soc* 135(31):11414–11416.
46. Shaikh M, Mohanty J, Singh PK, Nau WM, Pal H (2008) Complexation of acridine orange by cucurbit[7]uril and beta-cyclodextrin: Photophysical effects and pK_a shifts. *Photochem Photobiol Sci* 7(4):408–414.