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Reversible Insulin Self-Assembly under Carbohydrate Control

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The biological properties of peptides and proteins are under significant influence by their molecular weights, which impact, among other things, diffusion rates and kidney-mediated clearances. The rate of diffusion, in turn, is proportional to the half-life of subcutaneously injected depots of peptide and protein drugs. Insulin provides a good illustration: Monomeric insulins are fast acting ($t_{1/2}$ 1 h),[1] and hexameric insulins are intermediary acting ($t_{1/2}$ 2−4 h); on the other hand, insulins that bind to high molecular weight proteins[2] or form soluble high molecular complexes[3] are long acting ($t_{1/2}$ > 10 h).

Peptide and protein protraction via delayed diffusion has often been pursued by drug entrapment in polymers.[4] Furthermore, stimuli-controlled drug release from polymer systems has been demonstrated with polymers whose swelling is, for example, glucose-dependent.[5] However, the practical use of polymers faces a number of difficulties such as biocompatibility, drug instability, or denaturation and increase in drug volumes.

We report here a novel concept for peptide and protein stimuli-controlled protraction by reversible formation of soluble high molecular weight self-assemblies, as exemplified by insulin self-assembly under control by D-sorbitol and D-glucose. Specifically, by equipping insulin with pairs of boronates and carbohydrates or other polyols, sufficient driving force can be provided for formation of soluble high molecular weight insulin hexamer−hexamer self-assemblies. The use of boronate−carbohydrate binding for the peptide self-assembly enables carbohydrate-mediated displacement of the binding and, hence, carbohydrate-controlled peptide release, Figure 1.

Insulin in formulation with zinc(II) yields insulin hexamers, which constitute the stored form of insulin in both the pancreas and drug formulations. When given subcutaneously to diabetes patients, hexameric insulin is absorbed mainly via dissociation to dimer and monomer, and the resulting drug half-life is approximately 2 h. This timing fits fairly well with the meal-related requirement for insulin but is insufficient in the basal need for insulin (24 h/day). Long-acting insulin preparations have been obtained by insulin coprecipitation with protamine or by precipitation via tuning of the isoelectric point of the peptide. However, protractions from soluble depots generally yield better day-to-day reproducibility of drug absorptions. Soluble insulin depots have been obtained by insulin acylation with lipophilic groups that either enable binding to high molecular-weight proteins[2] or impose formation of soluble high molecular weight insulin self-assemblies.[3] However, it would be desirable if the insulin rate of absorption could be controlled by outside factors such as glucose or other small molecules that could be applied as meal-related release factors.

Boronates are known to bind to 1,2-diols such as carbohydrates in aqueous solution with affinities in the mM to μM range (K_d).[6] However, because simple boronic acids possess pK_a values of approximately 8.5, the binding is weak at the physiological pH of 7.4. Luckily, this problem can be corrected by use of electron-withdrawing groups,[7] whereby the pK_a of aryl boronic acids can be tuned to 7.4 or lower.

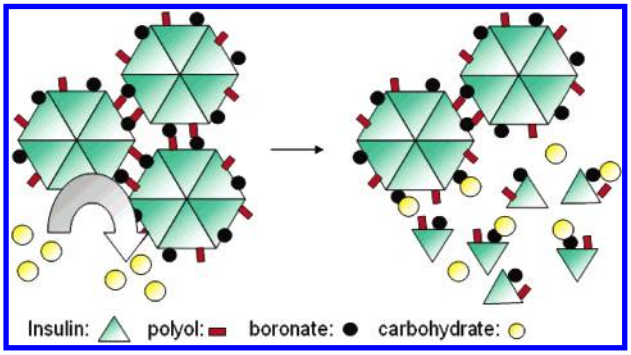
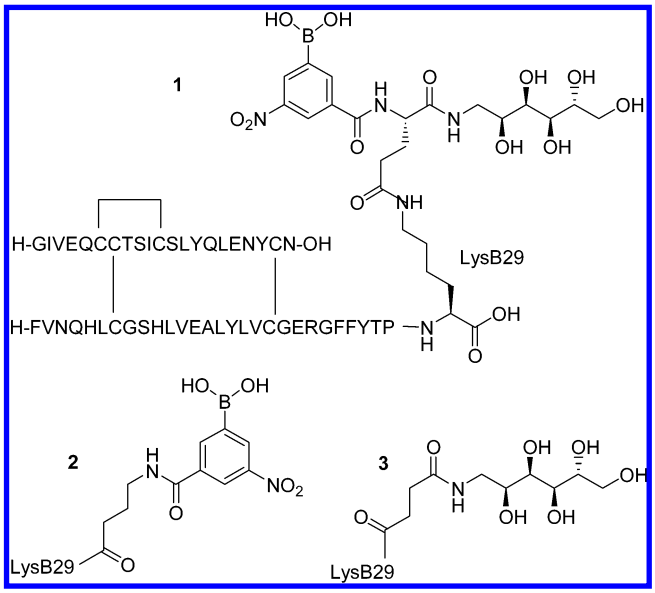


Figure 1. Illustration of insulin self-assembly under carbohydrate control.

Insulin **1** was prepared as described in Supporting Information. Insulins **2** and **3** were similarly prepared and used as controls. The required building blocks were attached to insulin LysB29 by acylation with succinimidyl esters at pH 10.5.[8] Importantly, to prevent the boronate−polyol pair of insulin **1** from binding internally within the insulin **1** monomer, the boronate−carbohydrate motifs were positioned on insulin via a common scaffold (L-glutamic acid).



The insulin receptor affinities of **1**−**3** were tested as previously described,[9] and the affinities were in the expected range relative to native insulin: 69, 122, and 85%, respectively. The LysB29 position is generally tolerant with respect to insulin receptor affinity and preservation of the overall properties of insulin.

The molecular weights of insulins **1**−**3** in zinc(II)/phenol formulations were analyzed by size-exclusion chromatography

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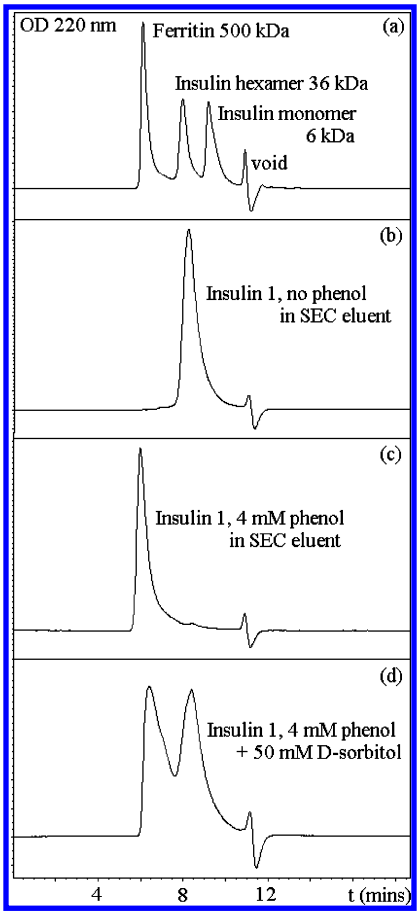

Figure 2. SEC analysis of insulin **1** with phenol and D-sorbitol in PBS buffer, pH 7.4, 37 °C.

(SEC) on a non-carbohydrate BioSep column. The standards being used were ferritin (500 kDa), Co(III) insulin hexamer (36 kDa), and AspB9-GluB27 insulin monomer (6 kDa), Figure 2a. Insulins **1**−**3** were dissolved at the standard conditions for clinical use of insulin: 600 μM insulin with 3 Zn(II)/hexamer, 16 mM phenol, 16 mM *m*-cresol, 7 mM phosphate, pH 7.4. The standard formulation contains glycerol as an isotonic agent, but due to the potential interference of this diol with boronate−carbohydrate binding, glycerol was replaced with 100 mM sodium chloride.

SEC analysis of insulin **1** initially showed only hexameric insulin peaks, Figure 2b. However, upon inclusion of 4 mM phenol in the SEC eluent, insulin **1** appeared as the desired high molecular weight complex, Figure 2c. Importantly, since the pH values of all formulations and buffers were adjusted to 7.4, the observation is not an effect of pH. Notably, since the control compounds, insulins **2** and **3**, appeared as hexamers in all SEC analysis (see Supporting Information), the boronate−glycol interaction is required for obtaining the observed self-assembly with insulin **1**.

The role of phenol in the self-assembly of **1** can be explained by considering the well-described phenol-dependent folding of insulin.[10] In formulation without phenol, the N-terminal of the B-chain of insulin is coiled (T-form),[11] whereas in phenol-containing formulation the same segment is α-helical (R-form, [phenol] > 2 mM).[11] Since self-assembly of insulin **1** is observed only in the presence of phenol, insulin **1** must apparently be in the R-form to allow the hexamers to pack in such geometry that the boronate−polyol groups can pair and hence effect formation of high molecular weight self-assembly. When phenol is present in the formulation only, the self-assembly cannot be detected by SEC, because phenol is weakly bound and removed during the SEC analysis. However, when phenol is present in the SEC eluent, the self-assembly is retained during the SEC analysis and can be detected. Light scattering measurements confirmed the SEC data, including the effect of phenol (see Supporting Information). Notably, phenol at low mM concentration is generally included in insulin formulations for human use, acting as both a preservative and a physical stabilizer, as the R-form is more stable than the T-form.[12]

The carbohydrate-controlled reversibility of the self-assembly of **1** was tested with D-sorbitol and D-glucose, which were added either to the formulation or to the SEC eluent. D-Sorbitol, which binds to boronates with a K_d of approximately 0.1 mM,[13] was found to disassemble the complex in a dose-dependent manner with an EC_{50} of 50 mM (Figure 2d). Equilibrium was reached as fast as the samples could be analyzed by SEC ($t_{1/2}$ is <5 min). Glucose, which binds to boronates with K_d of approximately 10 mM,[6] was not able to effect the disassembly of insulin **1**. However, by formulating **1** as a 1:1 mixture with DesB30 insulin, it was possible to observe an effect with glucose. Not surprisingly, the extent of self-assembly of the 1:1 insulin **1**/DesB30 mixture was less pronounced than with neat **1** (60 vs >95%). When glucose was included in the samples, there was a dose-dependent erosion of the self-assembly, and, for example, 100 mM glucose yielded 53% self-assembly.

In conclusion, the reported work illustrates a novel concept for peptide or protein protraction by soluble reversible self-assembly via polar interaction. Furthermore, the extent of self-assembly can be controlled by the addition of small molecules such as carbohydrates. The use of external polymers as diffusion modulators in drug protraction can thus be circumvented. The concept has been demonstrated for insulin **1** using boronates−polyols as recognition pairs and SEC-based detection of the soluble high molecular weight self-assemblies under control by D-sorbitol or D-glucose. Notably, the geometry of protein−protein packing appears to be critical in obtaining self-assembly. In the present case, insulin R-folding and hence the presence of phenol is required. The overall concept should be applicable to other peptides or proteins, although there will be a requirement for inherent formation of peptide or protein oligomers. Oligomer formation is, however, quite common for peptides and proteins or may be accessible by protein engineering.[14]

Supporting Information Available: Procedures for synthesis of insulin **1** and SEC analysis of insulins **1**−**3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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