DOI: 10.1002/cmdc.200700239

Vitamin B_{12} as a Carrier for the Oral Delivery of Insulin

Amanda K. Petrus,[a] Anthony R. Vortherms,[a] Timothy J. Fairchild,*[b] and Robert P. Doyle*[a]

The improved ease of insulin administration is considered important for attaining tighter glycemic control and better long-term clinical outcomes for people with diabetes mellitus (DM). A noninvasive insulin delivery route is therefore a major goal in the treatment of DM.[1,2] While a number of noninvasive strategies have been explored for the delivery of exogenous insulin, including: inhalation (intranasal and pulmonary), across the skin (transdermal), or orally via the gastrointestinal tract (GIT; oral–enteric),[3,4] each of these routes has limitations such as poor bioavailability, risk of concomitant pathogen uptake, abnormal lung function with extended use, and difficulty administering controlled dosage.[1] Of these techniques, delivery of insulin by inhalation has emerged as a promising alternative to multiple subcutaneous injections.[4–7] Inhalation is associated with a faster onset of action than subcutaneous injections and has demonstrated satisfactory reproducibility in all pharmacokinetic and pharmacodynamic parameters tested.[1,5] However, this route of administration is likely to be suitable only for the delivery of short-acting insulin analogues, and as such, will be most effective in combination with a basal/bolus insulin therapy.[5,7]

Although tight glycemic control is commonly achieved using basal/bolus insulin therapy involving intermediate- or long-acting insulin for basal control and multiple injections of pre-prandial short-acting insulin throughout the day,[8] patient acceptance of such a regime has, in the past, been slow.[9] The most likely cause for the slow patient acceptance is the burden associated with multiple daily injection.[10,11] A recent study also investigated the efficacy of incorporating pre-meal inhaled insulin versus subcutaneous regular insulin as part of a basal/bolus insulin regime and found that the inhaled insulin may provide an alternative for the management of type 1 DM when used in combination with an intermediate- or long-acting insulin for basal control.[7] Therefore, development of a noninvasive delivery route for intermediate- or long-acting insulin analogues may result in an entirely noninvasive delivery system for insulin using a basal/bolus regime.

[a] *A. K. Petrus, A. R. Vortherms, Prof. R. P. Doyle*
Department of Chemistry, Syracuse University
1-014 Center for Science and Technology, Syracuse, NY 13244-4100 (USA)
Fax: (+1) 315-443-3584
E-mail: rpdoyle@syr.edu

[b] *Prof. T. J. Fairchild*
Department of Exercise Science, Syracuse University
230 Huntington Hall, Syracuse, NY 13244 (USA)
Fax: (+1) 315-443-2258
E-mail: tjfairch@syr.edu

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

The two major limitations related to successful oral–enteric delivery are, and remain: 1) proteolysis in the GIT and 2) absorption at the intestinal enterocytes.[1,12] To address these problems we decided to use the dietary uptake pathway of vitamin B_{12}; mammals have an active transport mechanism in the GIT for the absorption and cellular uptake of the relatively large vitamin B_{12} molecule (Figure 1).[12]

Vitamin B_{12} first binds to haptocorrin, a salivary enzyme that protects and transports B_{12} through the stomach and into the small intestine. The B_{12} then binds to intrinsic factor (IF) and proceeds down the small intestine, where the complex binds to the IF receptor on the ileum wall. The IF–B_{12} receptor complex then undergoes endocytosis, releasing B_{12} into the blood serum, where it becomes bound to transcobalamin(II) (TCII). Conjugation to B_{12} may potentially protect bound proteins from digestion (protected by B_{12} uptake proteins) and also facilitate their internalization and transport into blood serum, thus overcoming the two major hurdles for oral insulin delivery mentioned above.[12] Previous research has demonstrated considerable success with the use of B_{12} to deliver proteins such as luteinizing-hormone-releasing hormone,[12] erythropoietin (EPO), and granulocyte stimulating factor.[13] Smaller agents such as tumor-targeting cytotoxins[14] have also been successfully delivered via this route. The use of B_{12} to deliver technetium-based radiopharmaceuticals has been postulated by Alberto and co-workers, who investigated conjugation of ^{99m}Tc through the cobalt center of the B_{12} corrin ring.[15] The key in all cases has been to couple so as not to disrupt the B_{12} uptake pathway, ensuring that the recognition of, and affinity for, the various B_{12} uptake proteins is not diminished.[14,16] We set out to systematically investigate B_{12}-based insulin delivery and we report herein our results on the synthesis, characterization, and purification of a new B_{12}–insulin conjugate with hypoglycemic properties as tested in vivo in streptozotocin (STZ)-induced diabetic rats.

By using 1,1′-carbonyldiimidazole (CDI), bovine insulin was directly conjugated at lysine 29 of the B strand to the ribose 5′-OH group of the α ligand of B_{12} to provide a carbamate-linked conjugate. Coupling of insulin through the B_{12} ribose 5′-OH group was performed because previous work had established that coupling at this position does not interfere with recognition by B_{12} uptake proteins.[12,14,16] The reason for this is illustrated in Figure 1 for B_{12} interaction with TCII. The B_{12} ribose unit is clearly solvent accessible and not involved in key recognition interactions. Studies of insulin conjugates and key residues involved in insulin receptor interactions suggested several positions, particularly on the B strand, where conjugation could be performed. Lysine B29 was chosen for ease of synthesis (the only two other ε-amines (both N termini) can be readily selectively protected, for example) and because it is known to be important for insulin oligomerization but not activity (the insulin monomer is considered the active species in vivo).

Despite modification at this point it is worth noting that experiments performed in any buffer containing high concentrations of divalent cations or with high ionic strength (such as phosphate-buffered saline) still resulted in significant insulin

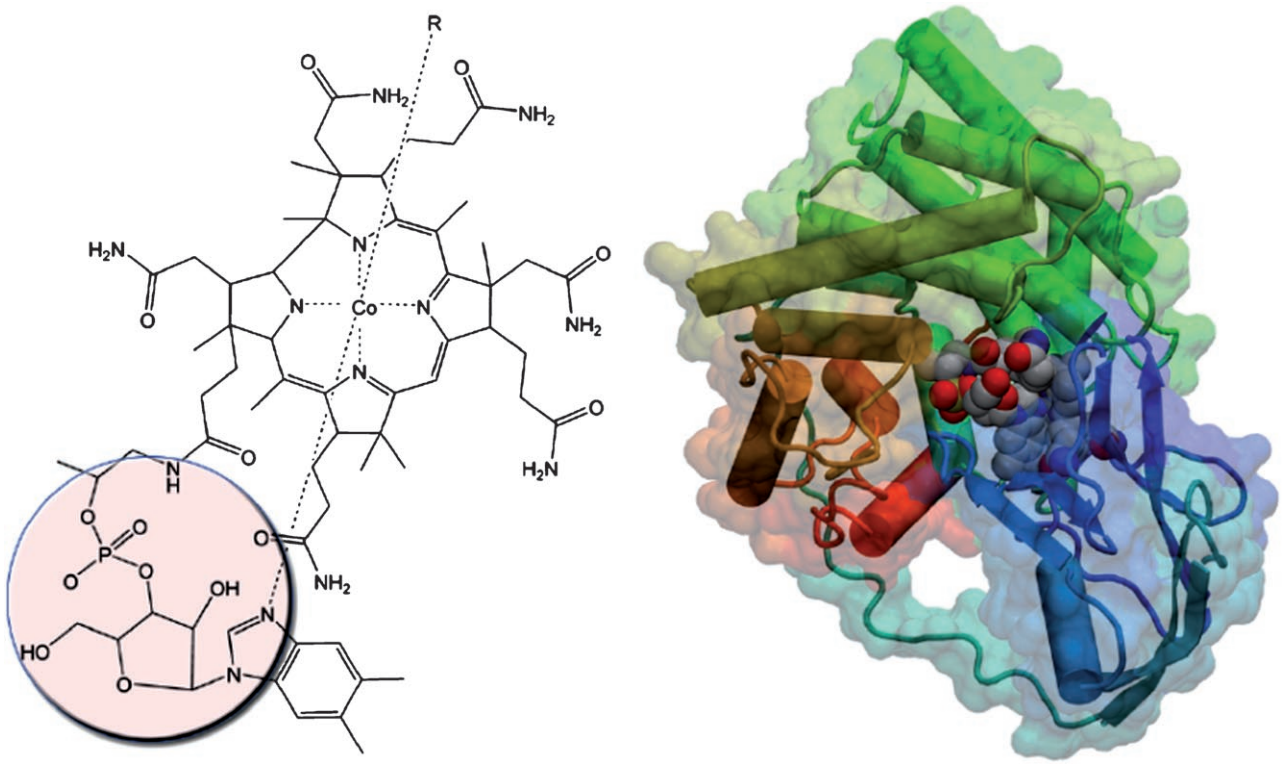

Figure 1. The structure of vitamin B_{12} (left) and the binding pocket of B_{12} in the TCII–B_{12} complex, with the solvent-accessible fragment of the B_{12} molecule visible. This is also shown on the left, circled.

polydispersity. This polydispersity is consistent with the presence of insulin oligomer formation (dimers and hexamers) as confirmed by velocity ultracentrifugation and C_{18} reversed-phase HPLC. These oligomers can aggregate and precipitate and also greatly hinder purification of the desired B_{12}–insulin conjugate. Mutation or modification of residues in the C-terminal region of the B strand of insulin, especially Thr B27, Pro B28, or Lys B29, has a dramatic effect on insulin association, greatly decreasing dimer and hexamer formation. Given that B_{12} is conjugated to insulin at Lys B29, oligomer formation must proceed through a process other than the formation of antiparallel β strands between two insulin C-terminal regions. This is likely through zinc-based interactions with residues such as His B10. What is clear is that conjugation alone to the C-terminal region does not prevent oligomerization and that conditions that promote such oligomerization must be avoided to obtain, in a facile manner, the desired B_{12}–insulin conjugate. This is best achieved in low molarity, chelex-washed HEPES buffer containing EDTA.

Coupling was attempted in the pH range 6.8–9.7 using coupling agents such as CDI and 1,1′-carbonyldi(1,2,4-triazole). CDI proved the most successful, especially if used in large excess (3- to 5-fold relative to B_{12}). More alkaline conditions produced better conjugation results, but prolonged exposure to such high pH resulted in precipitation, presumably due to insulin aggregation or deamination. As a result, upon completion of coupling at pH 9.7, dialysis was performed in 50 mM HEPES at pH 6.8 to both remove impurities such as CDI and unreacted B_{12}, and to bring the pH to a range in which these problems are minimized. Purification was carried out by dialysis to remove reagents of molecular weight <3500 Da, followed by anion-exchange chromatography to remove residual unconjugated insulin (Figure 2).

Velocity ultracentrifugation experiments were performed to verify the presence of only one species in the final purified sample (Figure 3). The species had an average molecular weight of $\sim 7000\ g\,mol^{-1}$ at 270 nm (**1** weighs $\sim 7200\ g\,mol^{-1}$) calculated from the experimentally derived sedimentation coefficient of 1.29×10^{-13} s. This is comparable with literature values of 1.25×10^{-13}, 1.65×10^{-13}, and 1.84×10^{-13} s for the insulin monomer ($5733\ g\,mol^{-1}$), dimer ($11\,466\ g\,mol^{-1}$), and hexamer ($34\,398\ g\,mol^{-1}$), respectively.[17] This indicates that a new, monodisperse sample has been obtained.

MALDI-ToF MS experiments in matrix with and without the reducing agent dithiothreitol (DTT) established that the desired conjugate had been synthesized and that the B_{12} is not conjugated to insulin on the A strand, which would have indicated failed protection of the terminal amines (Figure 4). No multiple conjugates (such as 2:1 B_{12}/insulin) were observed by MALDI-ToF or SDS-PAGE. This was further supported by velocity ultracentrifugation experiments.

Melting temperature circular dichroism studies (222 nm) confirm the insulin is still folded, resulting in a melting temperature at $\sim 65\,^{\circ}C$, similar to unconjugated insulin controls (see Supporting Information). Electronic absorption analysis shows maxima consistent with both the presence of B_{12} and insulin (see Supporting Information), and the peak at 361 nm ($\varepsilon = 27\,500\ M^{-1}\,cm^{-1}$) was used to calculate solution concentra-

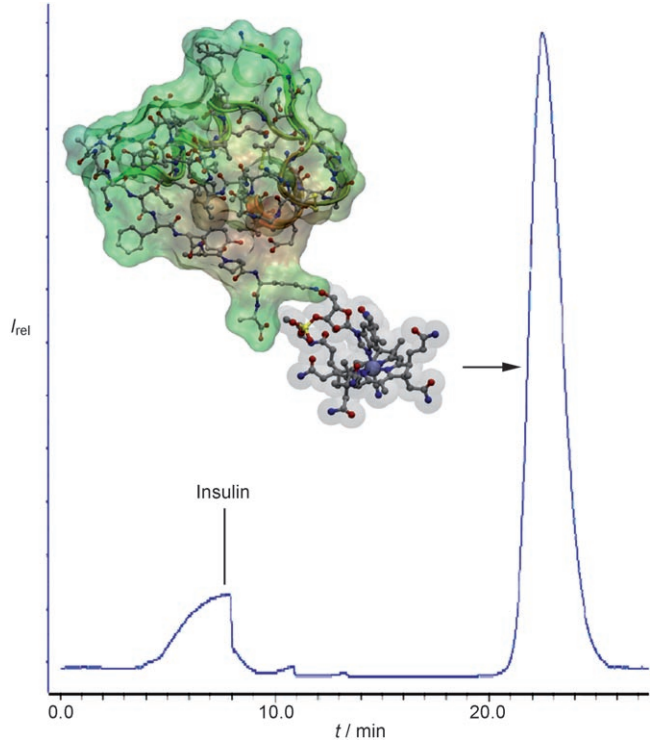

Figure 2. Anion-exchange (DEAE) chromatogram showing separation of insulin from **1**. The structure of **1** is shown with coupling of B_{12} and insulin at the ribose 5′-OH group and lysine B29 residue, respectively.

tion.[18] The B_{12}–insulin conjugate has been prepared at concentrations up to 27 μM. Spectrophotometric in vitro binding studies with IF confirm that the key enzyme in the B_{12} uptake pathway recognizes the B_{12}–insulin conjugate (see Supporting Information).

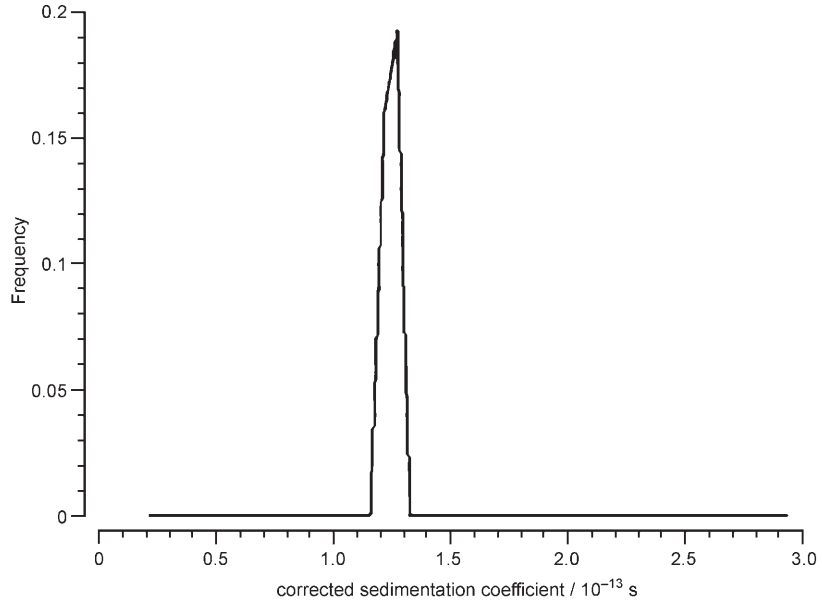

Figure 3. Velocity ultracentrifugation plot for purified **1** in 50 mM HEPES buffer, run at 40000 rpm at 10 °C and monitored at 270 nm; sedimentation coefficient: 1.285×10^{-13} s.

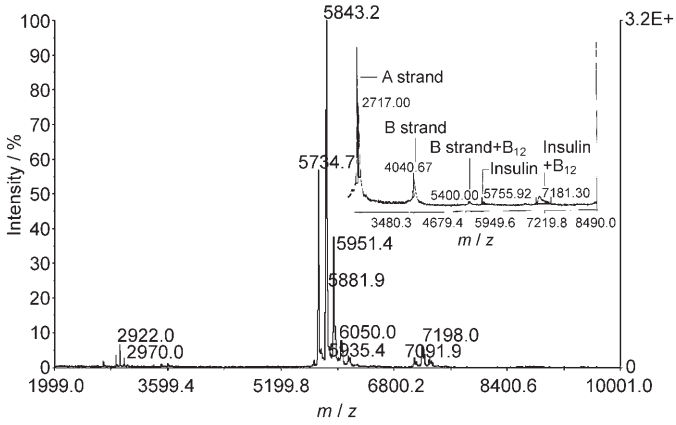

Figure 4. MALDI-ToF MS of **1**: Data for B_{12}–insulin in matrix containing no DTT and 10 mM DTT (inset). DTT reduces the disulfide links between both insulin strands. Both traces show a mix of "free" insulin control and B_{12}–insulin conjugate: (*m*/*z*): [M^+] for free insulin at 5734, **1** at 7091.9; with 10 mM DTT (*m*/*z*): [M^+] insulin A strand at 2717.00, B strand at 4040.67, B strand+B_{12} at 5400.00, insulin at 5755.92 and insulin+B_{12} at 7181.30. Note the presence of B_{12} bound only to the insulin B strand with no A strand–B_{12} observed.

To examine the in vivo efficacy of the B_{12}–insulin conjugate, blood from the STZ-induced diabetic rat model was sampled by jugular catheter prior to and subsequent to oral administration of the B_{12}–insulin conjugate and was compared with the blood glucose response following administration of an equimolar solution of free insulin (Figure 5). Prior to administration of compounds via oral gavage, fasting (>4 h) blood glucose levels confirmed that rats were hyperglycemic (15.6 ± 0.8 mmol L^{-1}; ± SEM) indicating that an insulin-deficient state had been achieved. Results identified that the B_{12}–insulin conjugate was associated with a 4.7-fold greater decrease in the area under the blood glucose curve ($p = 0.056$) relative to the blood glucose response to the administration of free insulin. To identify whether the corresponding change in blood glucose concentration is mediated by a B_{12}-dependent uptake pathway, the blood glucose concentration in response to administration of the B_{12}–insulin conjugate was compared with the blood glucose response to an identical dose of the B_{12}–insulin conjugate dissolved in 10^5-fold excess B_{12} (Figure 4). There was a significant ($p = 0.022$) decrease in the blood glucose response when the B_{12}–insulin conjugate was dissolved in 10^5-fold excess B_{12}. It is worth noting that the presence of excess B_{12} did not result in oligomerization of the conjugate as followed by analytical HPLC.

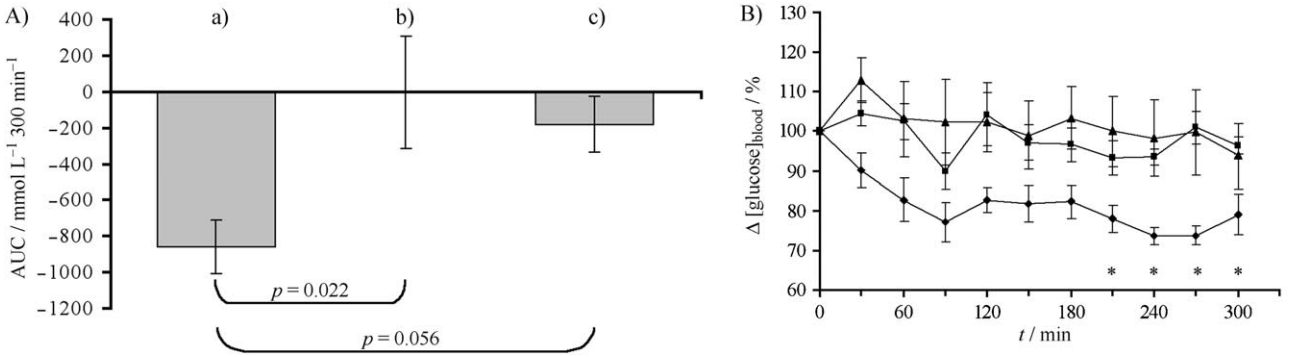

Figure 5. Blood glucose response in the STZ-induced diabetic rat model following administration of either the B_{12}–insulin conjugate ($n=7$); B_{12}–insulin dissolved in 10^5-fold excess B_{12} ($n=4$); or free insulin ($n=5$). A) Area under the blood glucose curve following administration of the three treatments: B_{12}–insulin (a), excess B_{12} (b), and insulin (c). Error bars represent SEM. B) Percent change in blood glucose in response to the administration of the three treatments: B_{12}–insulin (◆), excess B_{12} (▲), and insulin (■). Asterisks represent a significant difference ($p<0.05$) from the pre-administration value ($t=0$ min) for the B_{12}–insulin conjugate only.

Results obtained from the oral administration of the B_{12}–insulin conjugate indicate that the conjugate is more effective than free insulin in decreasing blood glucose levels (Figure 4). Furthermore, in comparison with results obtained in the B_{12}-excess trials, it is clear that the glucose-lowering effects of the B_{12}–insulin conjugate are mediated by the B_{12} uptake mechanism. The nadir in the blood glucose response in the present study occurred at 4 h, which is consistent with previous pharmacokinetic experiments with orally administered radiolabeled B_{12}.[19]

Given that excessive blood sampling may stimulate the release of catecholamines, the experiments were terminated by 5 h post administration. However, based on the blood glucose response curve, it is very likely that the hypoglycemic activity of the B_{12}–insulin conjugate could extend beyond the 5-h recording phase adopted in the present study. This is most likely due to the delayed uptake of the B_{12}–insulin conjugate,[19] but an extended half-life of the B_{12}–insulin conjugate relative to native insulin (10 min),[17] similar to transferrin-bound conjugates,[20] cannot be ruled out. Moreover, the role of enterohepatic recirculation of B_{12} conjugates requires further investigation because we postulate that this pathway may aid in prolonging the half-life of the B_{12}–insulin conjugate.[21]

The most remarkable finding of this study was the hypoglycemic response to the oral administration of the B_{12}–insulin conjugate and its dependence on the B_{12} uptake mechanism. However, while the clinical significance of this conjugate requires further investigation, it must be noted that the B_{12} uptake capacity in humans is limited to approximately 1–2 μg per dosage and as such, the amount of peptide that can be introduced through the B_{12} pathway is limited.[22] This limitation could be counteracted by multiple dosing (ileal IF receptors recycle every 30 min)[23] or by conjugating multiple insulin molecules to B_{12}. Indeed, the conjugation of multiple insulin molecules is a likely therapeutic preference given the versatility of this system that will allow extensive modifications with conjugation at various insulin residues at various B_{12} sites (both designed to minimize disruption of uptake and receptor recognition) and through linkers of various lengths; the ultimate aim is to optimize the uptake versus activity relationship between B_{12} and insulin for greatest activity and in vivo residency.

In conclusion, the improved ease associated with the noninvasive delivery of insulin is likely to yield stricter control of blood glucose levels and better clinical outcomes in individuals with DM. Herein we present an oral insulin delivery mechanism that has proven in vivo efficacy, is highly adaptive from a chemistry standpoint, and presents potential future clinical relevance as part of a noninvasive basal/bolus insulin therapy. Structure–function analysis will indicate whether such modification will impact in vivo glucose response. Derivatives (based on the conjugate described herein) are currently being prepared and characterized in vitro and in vivo to address this point; patent pending.[24]

Experimental Section

The protection of insulin was reported previously.[20] This procedure, slightly modified, is as follows: Insulin (25 mg, 4.3 μmol) was dissolved in 5 mL HEPES buffer (50 mM) with 25 mM EDTA. The solution was adjusted to pH 6.8–6.9 with 1 M sodium carbonate. A tenfold molar excess of dimethylmaleic anhydride (DMMA; 5 mg, 43 μmol) was dissolved in 1 mL DMSO. One third of the DMMA solution was added to the insulin, and the pH was adjusted back to 6.8–6.9 with 1 M HCl. The insulin was slowly rotated at 4 °C for 30 min. The pH was checked at the end of the 30 min and adjusted as before. The remaining two thirds of the DMMA solution were then added in the same fashion. The pH was again checked to be between 6.8 and 6.9, and the solution was allowed to rotate at 4 °C overnight. The protected insulin was dialyzed, with gentle stirring at 4 °C, against 50 mM HEPES buffer with 25 mM EDTA. Buffer (1 L) was changed every 3–5 h for a total of 4 L of buffer.

Activation of B_{12} and reaction with protected insulin: B_{12} (12 mg, 8.6 mol) was dissolved in 2 mL of dry DMSO. A fivefold molar excess of CDI (7 mg, 43 mol) was dissolved in DMSO and added to the B_{12} solution. The reaction was stirred at 35 °C for 2 h. 10 mL of dry diethyl ether was then added. The resulting red precipitate was collected by centrifugation and washed with another 10 mL of dry diethyl ether. The activated B_{12} was then added to the protected insulin and rotated at 4 °C overnight. The reaction was then dialyzed against 1-L volumes of HEPES buffer (50 mM, pH 7.4) until

the external buffer became clear. The solution inside the dialysis tubing remained pink at this point. Bringing the pH back down to 7.4 is important here to avoid insulin aggregation and/or deamination, which can occur at higher alkaline pH over prolonged periods. To test for the presence of residual B_{12} each liter of dialysis buffer was reduced to 1 mL in vacuo, and electronic absorption spectroscopy and electrospray mass spectrometry was performed. Once the presence of B_{12} was no longer observed (typically after 4×1-L changes), the pink solution remaining inside the dialysis tubing was then used for subsequent experiments. Ion-exchange chromatography to remove unreacted insulin was performed on a GE Akta Prime Plus system. A 5-mL HiTrap DEAE FF was loaded with 2.5 mL of dialyzed reaction. Unreacted insulin eluted with 100% water with a red fraction of **1** eluting with 50% 0.5 M NaCl. SDS-PAGE confirmed the presence of insulin, and the fraction was characterized by MALDI-ToF mass spectrometry and ultracentrifugation to verify a single species corresponding to B_{12}–insulin. UV/Vis: $\lambda = 360.0$, 411.9, 545.0 nm, all concentrations calculated using $\varepsilon_{360.0} = 27\,291\ M^{-1}cm^{-1}$; MALDI-ToF MS (*m/z*): $[M^+]$ for free insulin at 5734, B_{12} conjugated to insulin at 7091.9; CD: $T_m = 65\,^{\circ}C$. Reactions run with 25 mg (4.4 mmol) bovine insulin typically yielded 25 μM 5-mL solutions of B_{12}–insulin; yield ~3% based on insulin. B_{12} and insulin can be recovered and used in subsequent conjugations.

Acknowledgements

We thank Syracuse University, Promega Biotechnology (Wisconsin, USA) and the iLEARN Program for funding.

Keywords: diabetes · insulin · oral delivery · streptozotocin · vitamins

[1] W. T. Cefalu, *Diabetes Care* **2004**, *27*, 239–246.
[2] L. Heinemann, A. Pfützner, T. Heise, *Curr. Pharm. Des.* **2001**, *7*, 1327–1351.
[3] D. J. Chetty, Y. W. Chien, *Crit. Rev. Ther. Drug Carrier Syst.* **1998**, *15*, 629–670.
[4] C. D. Saudek, *Endocrinol. Metab. Clin. North Am.* **1997**, *26*, 599–610.
[5] V. P. Guntur, R. Dhand, *Respir. Care* **2007**, *52*, 911–922.
[6] J. S. Skyler, L. Jovanovic, S. Klioze, J. Reis, W. Duggan, *Diabetes Care* **2007**, *30*, 579–585.
[7] J. S. Skyler, R. S. Weinstock, P. Raskin, *Diabetes Care* **2005**, *28*, 1630–1635.
[8] A. Salsali, M. Nathan, *Am. J. Ther.* **2006**, *13*, 349–361.
[9] J. C. Zgibor, T. J. Songer, S. F. Kelsey, *Diabetes Care* **2000**, *23*, 472–476.
[10] E. D. Mollema, F. J. Snoek, R. J. Heine, H. M. van der Ploeg, *Diabetic Med.* **2001**, *18*, 671–674.
[11] A. Zambanini, R. B. Newson, M. Maisey, M. D. Feher, *Diabetes Res. Clin. Pract.* **1999**, *46*, 239–246.
[12] G. J. Russell-Jones, S. W. Westwood, P. G. Farnworth, J. K. Findlay, H. G. Burger, *Bioconjugate Chem.* **1995**, *6*, 34–42.
[13] G. J. Russell-Jones, S. W. Westwood, A. D. Habberfield, *Bioconjugate Chem.* **1995**, *6*, 459–465.
[14] A. M. Mitchell, A. Bayomi, E. Natarajan, *Enzymatic Mechanisms, Vol. 27* (Eds. P. A. Frey, D. B. Northrop), Ios, Amsterdam, **1999**, 150–154.
[15] S. Kunze, F. Zobi, P. Kurz, B. Spingler, R. Alberto, *Angew. Chem.* **2004**, *116*, 5135–5139; *Angew. Chem. Int. Ed.* **2004**, *43*, 5025–5029.
[16] H. P. C. Hogenkamp, D. A. Collins, F. G. West, C. B. Grissom, *Chemistry and Biochemistry of B_{12}* (Ed. R. Banerjee), Wiley, New York, **1999**, 385–410.
[17] F. Tietze, H. Neurath, *J. Am. Chem. Soc.* **1953**, *75*, 1758–1760.
[18] A. O. Hill, J. M. Pratt, R. J. Williams, *J. Chem. Soc.* **1965**, *46*, 2859–2865.
[19] a) J. Alsenz, G. J. Russell-Jones, S. Westwood, B. Levet-Trafit, P. C. de Smidt, *Pharm. Res.* **2000**, *17*, 825–832; b) J. D. Bagnato, A. L. Eilers, R. A. Horton, C. B. Grissom, *J. Org. Chem.* **2004**, *69*, 8987–8996.
[20] N. J. Kavimandan, E. Losi, J. J. Wilson, J. S. Brodbelt, N. A. Peppas, *Bioconjugate Chem.* **2006**, *17*, 1376–1384.
[21] S. Kanazawa, V. Herbert, B. Herzlich, G. Drivas, C. Manusselis, *Lancet* **1983**, *321*, 707–708.
[22] R. G. Jones, V. Ilic, D. H. Williamson, *Biochem. J.* **1984**, *220*, 455–460.
[23] J. A. Robertson, N. D. Gallagher, *Gastroenterology* **1985**, 88, 908–912.
[24] R. P. Doyle, *Vitamin B_{12} conjugated to biologically active peptides/proteins*, Patent filed: March 5, **2007**.

Received: September 4, 2007
Published online on September 25, 2007

