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Hydrogel Drug Delivery System Using Self-Cleaving Covalent Linkers for Once-a-Week Administration of Exenatide

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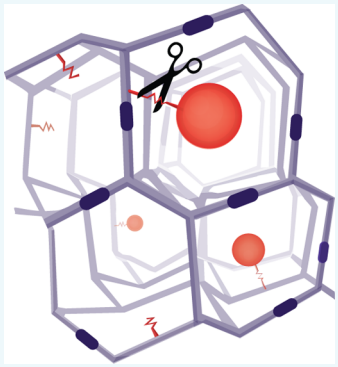
Supporting Information

ABSTRACT: We have developed a unique long-acting drug-delivery system for the GLP-1 agonist exenatide. The peptide was covalently attached to Tetra-PEG hydrogel microspheres by a cleavable β-eliminative linker; upon s.c. injection, the exenatide is slowly released at a rate dictated by the linker. A second β-eliminative linker with a slower cleavage rate was incorporated in polymer cross-links to trigger gel degradation after drug release. The uniform 40 μm microspheres were fabricated using a flow-focusing microfluidic device and in situ polymerization within droplets. The exenatide-laden microspheres were injected subcutaneously into the rat, and serum exenatide measured over a one-month period. Pharmacokinetic analysis showed a $t_{1/2,\beta}$ of released exenatide of about 7 days which represents over a 300-fold half-life extension in the rat and exceeds the half-life of any currently approved long-acting GLP-1 agonist. Hydrogel−exenatide conjugates gave an excellent Level A in vitro−in vivo correlation of release rates of the peptide from the gel, and indicated that exenatide release was 3-fold faster in vivo than in vitro. Pharmacokinetic simulations indicate that the hydrogel−exenatide microspheres should support weekly or biweekly subcutaneous dosing in humans. The rare ability to modify in vivo pharmacokinetics by the chemical nature of the linker indicates that an even longer acting exenatide is feasible.

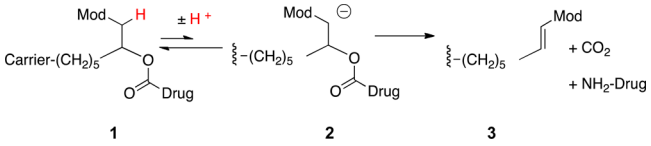


INTRODUCTION

Exenatide, a 39-amino-acid peptide from the Gila monster, is a potent agonist of the glucagon like-1 (GLP-1) receptor, and has glucoregulatory actions similar to GLP-1. In the human, exenatide has a significantly longer plasma lifetime than GLP-1, but nevertheless has a $t_{1/2,\beta}$ of only ∼2.5 h. It is marketed for type 2 diabetes as Byetta, and is administered subcutaneously (s.c.) twice a day. There is both demand for and benefit in long-acting forms of GLP-1 agonists,[1−3] and agonists have been developed that are administered s.c. once a day (Victozia, Lyxumia) or once a week (QWK) (Bydureon, Tanzeum, and Trulicity are approved and Semaglutide is in late-stage trials). There is also an implantable osmotic pump, ITCA 650, in late clinical trials that delivers exenatide over 6 to 12 months;[4] however, the level of patient-acceptance of an implantable device vs simple s.c. drug injections remains uncertain.

We have recently developed a chemical approach for half-life extension of drugs; here, a drug is covalently attached to a long-lived macromolecule through a β-eliminative linker that is slowly cleaved to release the native drug.[5,6] The carrier may be a circulating macromolecule such as polyethylene glycol (PEG) for half-life extensions of up to one or two weeks,[5] or noncirculating hydrogel depots, for half-life extension of weeks to months.[6] In this approach, a macromolecular carrier is attached to a linker that is attached to a drug via a carbamate group (**1**; Scheme 1); the β-carbon has an acidic carbon−hydrogen bond (C−H) and also contains an electron-withdrawing pK_a "modulator" (Mod) that controls the acidity of that C−H. Upon hydroxide ion-catalyzed proton removal to give **2**, a rapid β-elimination occurs to cleave the linker-carbamate bond and release the free drug and a substituted alkene **3**. The rate of drug release is proportional to the acidity of the proton which is controlled by the chemical nature of the modulator; thus, the drug release rate is controlled by the modulator. When using noncirculating hydrogel carriers, we also incorporate slower-cleaving β-eliminative linkers in the cross-links so polymer degradation can be triggered after drug release.[6]

Scheme 1



We previously reported a releasable PEGylated exenatide that had an estimated $t_{1/2,\beta}$ of ∼50 h in the human.[5] However, as with any circulating conjugate, the renal elimination rate of the conjugate limits the potential half-life extension. Thus, a noncirculating hydrogel−exenatide conjugate was prepared that showed a $t_{1/2,\beta}$ of ∼80 h. The problems of this conjugate were that the $t_{1/2,\beta}$ was not sufficiently long for QWK administration, s.c injection required a large 18 gauge needle, and the hydrogel was not biodegradable. Recently, we reported a drug delivery

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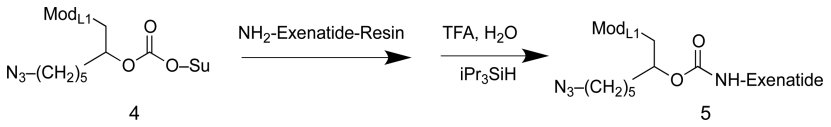
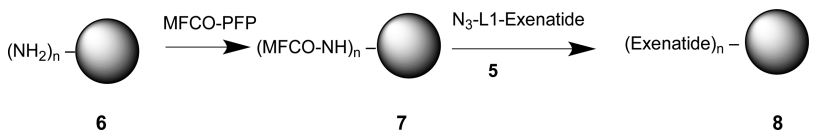
Scheme 2



Scheme 3



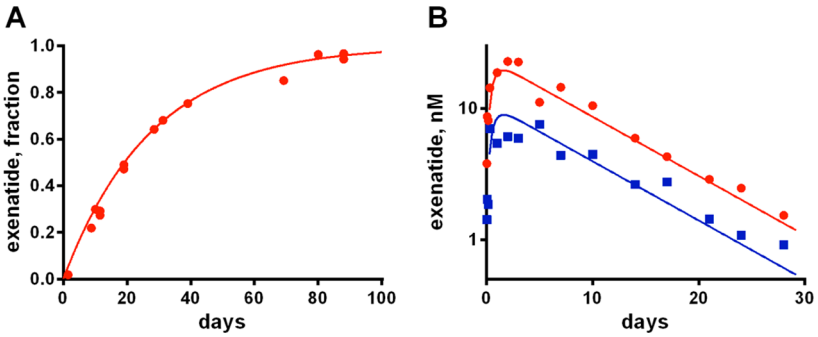


Figure 1. Release of exenatide from hydrogel microsphere conjugates. (A) In vitro release determined under accelerated cleavage conditions of pH 8.4, 37 °C, and shown after extrapolation to pH 7.4 by $k_{pH\ 7.4} = k_{pH} \times 10^{(pH-7.4)}$.[5,6] (B) Serum exenatide levels after s.c. injection of rats with hydrogel-exenatide microspheres. Gels containing 1.2 μmol (blue ■) and 2.7 μmol (red ●) exenatide/kg showed C_{max} values of 8.9 and 20 nM, respectively. Lines are the best fit to data using simplex optimization to models giving a $t_{1/2,\beta}$ of 160 ± 13 (SE) for the hydrogel–exenatide conjugates.

system (DDS) based on a Tetra-PEG hydrogel[7] carrier that is injectable, biocompatible, and biodegradable.[6]

In the present work, we have attached exenatide via a β-eliminative linker to degradable 40 μm Tetra-PEG hydrogel microspheres and demonstrated a $t_{1/2,\beta}$ for released drug of about 7 days in the rat. Simulations of the pharmacokinetics (PK) in humans suggest that this noncirculating hydrogel–exenatide conjugate should maintain therapeutic levels of the drug with weekly or biweekly s.c. administration.

■ RESULTS

The general approach used for preparing the hydrogel–exenatide conjugate DDS was to prepare an N-terminal azido-linker (L1)-exenatide,[5] and then couple it by strain-promoted azide–alkyne cycloaddition (SPAAC) to monofluorocyclooctyne (MFCO)-derivatized hydrogel microspheres containing cleavable cross-links (L2).[8]

Chemistry. In a modification of a reported method,[5] exenatide was synthesized by SPPS using Fmoc/tBu chemistry and reacted on-resin at the α-amine group with 7-azido-1-(methylsulfonyl)-2-heptyl succinimidyl carbonate (**4**; N$_3$-L1-[MeSO$_2$-]-HSC)[5] in DMF containing *N*-methyl morpholine (Scheme 2); here, scrupulous removal of amines from DMF and use of a weak base were important to avoid β-elimination of the linker. In early experiments, we observed that when the peptide-resin and blocking groups were cleaved with 95:5 TFA:H$_2$O, 5% DTE, the azide moiety of the linker was reduced. Subsequently, we found that reaction of the peptide-resin with 95:5 TFA:H$_2$O containing 5% triisopropylsilane instead of DTE provided good yields (~60%) of the N$_3$-L1[MeSO$_2$-]-exenatide **5**.

The chemistry used for amine-derivatized Tetra-PEG hydrogel production is a slight modification of that previously reported.[8] Here, we created a prepolymer in which PEG$_{20\ kDa}$-(NH$_2$)$_4$ was first acylated with an N$_3$-L2-[(MeOCH$_2$CH$_2$)$_2$NSO$_2$-]-Lys(Boc)-HSE, followed by removal of the Boc group. The azide groups of this prepolymer were then reacted with PEG$_{20\ kDa}$-(MFCO)$_4$ by SPAAC to provide the amine-derivatized hydrogel microspheres containing cleavable cross-links.

Microsphere Production. We developed a method to prepare the hydrogel as 40 μm amine-derivatized microspheres **6** in a flow-focusing microfluidic device (Figure S1). The two prepolymer solutions are introduced through a Y-junction to mix in the device; the resulting aqueous stream is sheared with an immiscible continuous phase to form droplets that undergo spontaneous polymerization into microgels. When operated continuously, the single 7-channel device used could produce almost 200 mL of swollen amino-microspheres per day, which is scalable by running multiple chips in parallel. These microspheres (a) can easily be chemically modified by acylation of the amine groups, (b) have cleavable β-eliminative cross-links to allow biodegradation or base-catalyzed degelation, and (c) can readily be injected through a 27-gauge needle. Initially, we produced these microspheres using fluorinated solvents and surfactants commonly employed in microfluidic droplet production.[9] Subsequently, the amine-derivatized microspheres were produced by a method that replaced these materials of unproven safety and high cost. We were able to substitute the fluorinated solvent, HFE-7500, as the continuous phase and wash solvent with decane. In place of the perfluorinated PEG-Krytox block copolymer surfactant, we used Abil-EM90 and polyglycerol polyresorcinoleate[10] which are extensively used in cosmetics and foods, respectively. Substitution of the

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fluorinated specialty reagents by these commodity chemicals also reduced the cost of materials by well over 100-fold.

The tendency of the hydrogel microspheres to adhere to vessel surfaces made them difficult to transfer, and differential solvent- and modification-dependent swelling made their quantitation problematic. Inspired by SPPS methodology, we constructed a syringe-based reaction vessel (Figure S2) that allowed aseptic introduction and removal of reagents, as well as chemical modification and washing without transfer or loss of microspheres; the vessel also permitted quantitative syringe-to-syringe transfer of the microsphere slurries. Using this assembly, TFA-sterilized[11] amino-microspheres **6** were acylated with MFCO-pentafluoro phenyl ester,[12] and the MFCO-microspheres **7** then coupled with the azido-linker-exenatide by SPAAC to give the exenatide-loaded microspheres **8** (Scheme 3).

Under accelerated base-catalyzed linker cleavage conditions[5,6] the Tetra-PEG hydrogel-exenatide microspheres showed an extrapolated in vitro release $t_{1/2}$ of 460 h for exenatide (Figure 1A) and a reverse gelation time (t_{RG}) of 200 days at pH 7.4, 37 °C. Previously, we empirically observed that in vitro rates of linker cleavage at pH 7.4, 37 °C, are ~2- to 3-fold slower than in vivo rates.[5] The total exenatide released corresponded to a loading of ~2 μmol peptide/mL packed microspheres.

PK in the Rat. Figure 1B shows the serum exenatide C vs t plots over 28 days after s.c. injection of microspheres containing 1.2 μmol and 2.7 μmol exenatide/kg in the rat.

The PK were modeled as three consecutive first-order reactions occurring in the subcutaneous (SC) or central (CC) compartments (Scheme 4).

Scheme 4

$$\text{Gel-Drug}_{SC} \xrightarrow{k_1} \text{Drug}_{SC} \xrightarrow{k_a} \text{Drug}_{CC} \xrightarrow{k_2} \text{elimination}$$

Here, k_1 and k_2 are the linker cleavage and exenatide elimination rate constants, respectively, and k_a is the rate constant for absorption of the released free exenatide into the central compartment. The model was first expanded to a triexponential integrated rate equation.[13] The fast elimination of free exenatide in the rat[14] relative to release from the hydrogel (k_1) and absorption from the s.c. compartment (k_a) allows the data to be described by the simpler two-exponential eq 1

$$[\text{Drug}]_{CC,t} = \text{Dose}\cdot Q\cdot(e^{-k_1 t} - e^{-k_a t}) \quad (1)$$

and when absorption is also fast relative to release

$$Q = \frac{F}{V_{SS}\cdot k_2}\cdot k_1 = \frac{F}{CL}\cdot k_1 \quad (2)$$

The PK analysis provides values for k_a and k_1, but not for bioavailability, F, volume of distribution, V_{ss}, or drug elimination rate, k_2, as these parameters are fit to data as the aggregate scaling factor Q.

The two data sets of Figure 1B, differing only in the initial dose, were simultaneously fit to eq 1 using a Nelder–Mead downhill simplex[15] with k_1, k_a, and Q as variables and the sum of residuals squared as error function. This analysis showed dose-proportional drug levels and provided estimates of $t_{1/2,a}$ = 8.7 h, $t_{1/2,1} = t_{1/2,\beta}$ = 160 h, and Q = 0.0092. Using eq 2, CL/F is estimated as 7.8 mL/kg-min, in good agreement with the reported value of 8.6 mL/kg-min.[16] The $t_{1/2,\beta}$ represents a 343-fold half-life extension over bolus exenatide in the rat.

Figure 2A shows excellent agreement of the cumulative in vitro release and in vivo absorption profiles for the Tetra-PEG hydrogel–exenatide conjugate with a $MeSO_2$-modulator, as well as the previously PEGA hydrogel–exenatide conjugate having a faster cleaving linker;[5] although only the higher dose of Figure 1 is shown, the lower dose matched as well. To obtain the in vivo–in vitro fits, the in vivo $t_{1/2,\beta}$ values are scaled to be 2.9-fold lower than the $t_{1/2}$ of in vitro release. Figure 2B shows the Level A in vitro–in vivo correlation (IVIVC) plots[17,18] of in vivo exenatide absorption vs in vitro release of the Tetra-PEG-exenatide conjugate (R^2 = 0.995) as well as PEGA hydrogel conjugate (R^2 = 0.993) from Figure 2A.[5] The data show that in vivo exenatide release from the hydrogels is well predicted by in vitro release studies, and that in vivo drug release is about 3-fold faster than in vitro release.

PK Simulations in the Human. We have described approaches for simulations of the PK of conjugates containing β-eliminative linkers in humans.[5] First, we simulate parameters for a single dose of the drug conjugate that would maintain a specified minimal plasma drug concentration, C_{min}, for the time interval desired in multiple dosing; then, steady state values are estimated by nonparametric superposition of sequential single dose simulations. Simulations of interspecies PK with

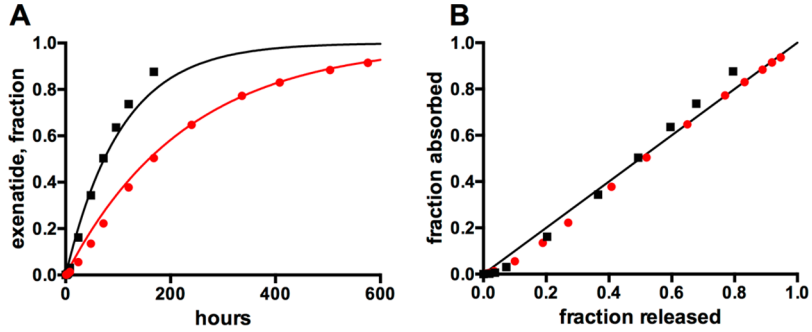

Figure 2. IVIVC of releasable hydrogel-exenatide conjugates. (A) Cumulative in vitro (lines, black, red) and in vivo (red ●, black ■) release from hydrogels with $MeSO_2$- (red, ●) and $ClPhSO_2$- (black, ■) modulators; in vitro release rates are scaled by an increase of 2.9-fold. In vivo absorption was calculated from data in Figure 1B and from Figure 3B in ref 5 using the FDA-recommended[18] deconvolution method of Nelson-Wagner.[19] (B) Level A correlation between in vitro release and in vivo absorption from hydrogels using $MeSO_2$- (red ●) and $ClPhSO_2$- (black ■) modulators for release. The line depicts a perfect correlation of absorbed and released exenatide.

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Table 1. Reported and Simulated PK Parameters of Long-Acting Exenatide in Humans at Steady State

drug (modulator)	dosing interval	Exenatide					
		dose, mg	$t_{1/2,\beta}$, h	C_{min}, nM	C_{max}, nM	C_{max}/C_{min}	AUC nM-h/wk
Bydureon[a]	QWK	2	NA	0.07	0.120	1.7	12
Simulations							
PEG-exenatide ($PhSO_2^-$)	QWK	1.3[b]	53[c]	0.07	0.67	9.6	49
Hydrogel-exenatide ($pClPHSO_2^-$)	QWK	0.9[b]	78[c]	0.07	0.28	4.0	27
Hydrogel-exenatide ($MeSO_2^-$)	QWK	0.6[b]	160	0.07	0.14	2.0	17
Hydrogel-exenatide ($MeSO_2^-$)	Q2WK	1.9[b]	160	0.07	0.29	4.1	54

[a]Bydureon PK parameters from refs 23,24. [b]Dose values for simulations of exenatide conjugates assume 100% bioavailability of exenatide. [c]$t_{1/2,b}$ of released exenatide taken from ref 5.

circulating PEG−drug conjugates have been reported,[6] but analogous approaches for simulations of noncirculating releasable conjugates have, to our knowledge, not been previously described.

For noncirculating carriers, the simulation requires knowledge of the in vivo linker cleavage rate, k_1, in one species—such as the rat—the k_1 in the human, and the parameters $t_{1/2,\beta}$ and V_{ss} of the released free drug in the human. Although k_1 values for these linkers have not yet been determined in humans, since linker cleavage rates are chemically controlled and are the same in the mouse, rat, and monkey,[5,20] it is reasonable to assume they are species independent. Estimation of the steady state dosing of a noncirculating carrier−drug conjugate needed to provide a given C_{min} of the drug at the end of a designated dose interval is performed as follows.

In cases of eq 1 where absorption of drug is fast relative to release from the hydrogel, i.e., $k_a \gg k_1$ in Scheme 4, after a short initial absorption phase eq 1 reduces to the single exponential eq 3.

$$[\text{Drug}]_{CC,t} = \frac{\text{Dose}\cdot F}{V_{SS}}\cdot\frac{k_1}{k_2}\cdot e^{-k_1 t} \tag{3}$$

Since $[\text{Drug}]_{CC,t} = C_{min}$ when $t = t_{min}$

$$\text{Dose}_{total} = C_{min}\cdot\frac{V_{SS}\cdot k_2}{F\cdot k_1}\cdot e^{-k_1 t_{min}} = C_{min}\cdot\frac{CL}{F\cdot k_1}\cdot e^{-k_1 t_{min}} \tag{4}$$

Here, Dose_{total} is the amount of drug on the carrier at initial dosing that is needed to achieve the desired steady state C_{min} at dose-interval time t_{min}. The drug remaining on the carrier at time t_{min}, $\text{Dose}_{remnant}$, is given by

$$\text{Dose}_{remnant} = \text{Dose}_{total}\cdot e^{-k_1 t_{min}} \tag{5}$$

The amount of drug required for steady-state dosing, Dose_{SS}, is the amount to be added to the remnant to reach Dose_{total}.

$$\text{Dose}_{ss} = \text{Dose}_{total} - \text{Dose}_{remnant} = \text{Dose}_{total}\cdot(1 - e^{-k_1 t_{min}}) \tag{6}$$

which by substituting eq 4 for Dose_{total} becomes

$$\text{Dose}_{ss} = C_{min}\cdot\frac{V_{ss}\cdot k_2}{F\cdot k_1}\cdot(e^{k_1 t_{min}} - 1) = C_{min}\cdot\frac{CL}{F\cdot k_1}\cdot(e^{k_1 t_{min}} - 1) \tag{7}$$

Since $V_{ss}\cdot k_2 = CL$, estimates may be made using either parameter in eqs 4 and 7. The number of doses to reach steady state, N, with a noncirculating carrier-linked drug can be estimated by eq 8, where f_{SS} is the fraction of the steady state C_{min} reached after the Nth dose. For example, if the dosing interval t_{min} is 168 h, and $t_{1/2,1}$ is 160 h, ∼95% steady state[21] C_{min} is reached after four doses.

$$N = -\frac{\ln(1 - f_{SS})}{\ln 2}\cdot\frac{t_{1/2,2}}{t_{min}} \tag{8}$$

For steady-state dosage estimations of hydrogel−exenatide conjugates in humans, we used $t_{1/2,\beta}$ and V_{ss} of the released exenatide of 2.4 h and 0.4 L/kg, respectively, which are values for bolus s.c. injected exenatide,[22] and targeted a C_{min} of ∼70 pM which is the exenatide C_{min} achieved with 2 mg weekly dosed Bydureon.[23,24] Steady-state values of exenatide released from weekly dosed Bydureon and simulated PK parameters of previously and presently reported exenatide conjugates are compared in Table 1.

We previously reported a circulating PEG−exenatide conjugate that released exenatide with an in vivo cleavage $t_{1/2}$ of 78 h.[5] Simulations indicated that in the human the $t_{1/2,\beta}$ of released exenatide should be 53 h, and that the conjugate could maintain 70 pM drug with once weekly administration of an exenatide dose similar to Bydureon. However, because of the relatively short in vivo $t_{1/2}$ for linker cleavage and concurrent elimination of ∼30−40% the PEG−peptide conjugate with $t_{1/2}$ ∼ 5 to 6 days,[25] utilization of the drug would be inefficient and C_{max} would be high. We therefore examined a noncirculating s.c.-administered hydrogel−exenatide conjugate that coincidentally also had $t_{1/2,\beta}$ of ∼80 h for exenatide but was not lost by elimination. As expected, the estimated dose required to maintain a 70 pM C_{min} was lower than those of either the PEG-conjugate or Bydureon, but C_{max} was 2.3-fold higher that of exenatide released from Bydureon.

By using the Tetra-PEG hydrogel-exenatide DDS described here with a linker having a longer cleavage $t_{1/2}$ of 160 h, exenatide concentrations of ≥70 pM should be maintained with weekly dosing of a hydrogel−exenatide conjugate containing only ∼0.6 mg exenatide, with a C_{max}, C_{max}/C_{min}, and AUC similar to Bydureon. This would require an injection of ∼0.1 mL of the microsphere−exenatide conjugate per 70 kg person. Simulations indicate that the same hydrogel-conjugate might also serve for biweekly dosing with quite acceptable dose and C_{max}.

DISCUSSION

In the present work, we describe the preparation and properties of a DDS in which exenatide is covalently attached to Tetra-PEG hydrogel microspheres by a releasable β-eliminative linker; upon s.c. injection, the exenatide is slowly released by a rate dictated by the linker. A second β-eliminative linker with a slower cleavage rate was incorporated in polymer cross-links to trigger gel degradation after the drug had been released.

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For synthesis, the azide-terminated linker was attached to the N-terminus of exenatide as the final coupling step in SPPS, followed by cleavage and purification of the azido-linker-exenatide. Separately, degradable Tetra-PEG amino-microspheres of 40 μm diameter were prepared in a microfluidic device, and derivatized with a cyclooctyne. Then, the azido-linker-exenatide was coupled to the cyclooctyne-microspheres by SPAAC to give the microsphere-exenatide DDS.

The exenatide-laden Tetra-PEG microspheres were injected s.c. into the rat at two doses, and serum exenatide was measured over a one month period. PK analysis showed high bioavailability and a $t_{1/2,\beta}$ of ~7 days, a ~340-fold half-life extension over bolus exenatide. This conjugate and another having a faster release rate both gave an excellent Level A IVIVC in the rat. The results showed that in vitro release serves as a surrogate for in vivo release, and indicates that the in vivo cleavage is about 3-fold faster than in vitro. Although the reason for the faster in vivo release rates is unknown, it cannot be due to foreign body responses to the s.c. microspheres—as suggested for PLGA-encapsulated exenatide[26]—since circulating PEG-conjugates containing these linkers show similar in vivo rate enhancements.[5]

Since the β-eliminative linker cleavage rate is species independent,[5,20] using known PK parameters of exenatide in the human, we could simulate the PK of the hydrogel DDS in the human. Results indicate that weekly s.c. dosing of only ~0.6 mg exenatide attached to the microspheres should maintain therapeutic levels of the drug with a PK profile similar to Bydureon, and biweekly administration of about 1.9 mg should maintain therapeutic levels of the peptide with acceptable C_{max} and peak-over-trough values. Although these doses were calculated assuming 100% bioavailability of the released exenatide, the assumption is justified by the high bioavailability of s.c. exenatide in the human.[27]

There are three current formats for half-life extension in marketed GLP-1 agonists—controlled release, peptide agonist fusions, and albumin binding; the benefits and harms of these have recently been reviewed.[28] Our releasable hydrogel-exenatide is most analogous to the QWK PLGA-controlled release formulation of exenatide, Bydureon, and derives substantial benefit from the large information-base derived from this DDS. Notably, extensive correlations of the PK of exenatide with its PD and toxicity provide a high degree of confidence that if we achieve the desired PK, we can attain the desired therapeutic outcome. Further, the strong PK–PD relationship of exenatide allows pathways that can significantly shorten the time to regulatory approval (i.e., the FDA 505b2 pathway).

The hydrogel–exenatide DDS has several benefits over Bydureon. First, the in vivo release profile of exenatide from the hydrogel DDS is much simpler and predictable than from the PLGA encapsulated formulation. Each dose of the former is followed by a simple first-order in vivo release of exenatide that allows easy estimation of drug levels upon multidosing. In contrast, a single dose of Bydureon shows an initial burst, a lag period, and then a period ~4 to 8 weeks after administration during which most of the exenatide is released;[29] achieving a desired steady-state drug level requires harmonized superpositioning of the irregular periods of drug release upon sequential doses of the polymer. Second, because the drug in the current DDS is covalently bound to the carrier, there is no burst effect due to loosely adhering or rapidly escaping drug. Third, whereas Bydureon must be stored dry and reconstituted shortly before injection through a 23 gauge needle, the Tetra-PEG hydrogels and linkers are stable at reduced pH as an aqueous suspension[5,6] and the 40 μm microspheres can be easily injected through a small-bore 27 gauge needle. Finally, unlike PLGA formulations that can significantly glycolate and lactoylate amines of an encapsulated peptide,[26,30] the current DDS releases the peptide in the form that is attached to the carrier.

The other approved once-weekly GLP-1 agonists consist of a fusion of the peptide with a long-lived circulating macromolecule (Abglutide, Dulaglutide), or a peptide agonist modified such that it binds to serum albumin (Semaglutide). With a $t_{1/2,\beta}$ of nearly 7 days, the released exenatide from our DDS has a lifetime comparable to or exceeding the approved long-acting GLP-1 agonists. Further, two unique features of the current DDS distinguish it from all others. First, a second peptide could be connected to the hydrogel–exenatide conjugate by the same β-eliminative linker to synchronize their in vivo half-lives. This should provide a simple method to create a dual-effector DDS functionally analogous to single peptides developed to have GLP1R-GCGR[31] or GLP1R-GIPR[25] co-agonism. Second, a unique feature of the current DDS is the ability to modify the half-life of the released drug by modifying the β-elimination rate of the linker. Thus, it should be feasible to create a DDS that delivers its cargo over a period of one month or longer. The preparation of such dual-effectors using the current hydrogel-exenatide DDS and ultra-long-acting hydrogel–exenatide conjugates are in progress.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00690.

The source of specialized materials is provided along with their use. Detailed synthetic and conjugation procedures are described as well as analytical procedures. Methods of preparing and conjugating hydrogel microspheres are also furnished. In vitro kinetic procedures are provided as are in vivo PK methods and analyses. (PDF)

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Notes

The authors declare the following competing financial interest(s): All authors are employees of and have equity in Prolynx.

■ ACKNOWLEDGMENTS

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Supplementary Information

Hydrogel drug delivery system using self-cleaving covalent linkers for once-a-week administration of exenatide

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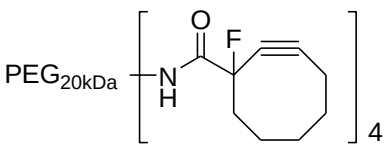
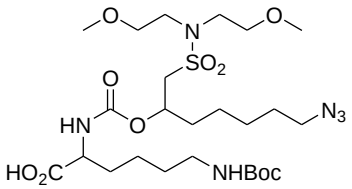
1. General. Amino-PEG reagents for microsphere preparation were from JenKem. HPLC used a Shimadzu Prominence UFLC equipped with an SPD-M20A diode array detector. RP-HPLC analysis used a Jupiter 5µ C18 300A 150 x 4.6-mm HPLC column (Phenomenex) with a 1 min isocratic flow of 20% MeCN-0.1% TFA followed by a 10 min linear gradient of 20- to 100% MeCN-0.1% TFA at 1 mL min^{-1}. UV analyses were performed using a Hewlett-Packard 8453 UV-Vis spectrophotometer. TNBS analysis of amines [1] and DBCO analysis of azides [2] were as described.

2. N^{α}-[1-(methylsulfonyl)-7-azido-2-heptyloxycarbonyl]-exenatide. The modified peptide was prepared by SPPS and on-resin N-terminal carbamoylation as previously described [1] with the following modifications: a) the DMF used was scrupulously freed of basic amines by treatment with Aldramine (Sigma-Aldrich); b) three equivalents N-methylmorpholine were used instead of N,N-diisopropyl-ethylamine in the attachment of linker to the N-terminus; and c) the N-carbamoyl-peptide was cleaved from the resin using 90% TFA, 5% H_2O and 5% triisopropylsilane for 3 hr at ambient temperature. RP-HPLC and DBCO-consumption azide analysis by UV [2] of the crude peptide indicated that ~60% of the A_{280} was the desired azido-linker-exenatide, which was purified by preparative-HPLC and characterized by LC/MS.

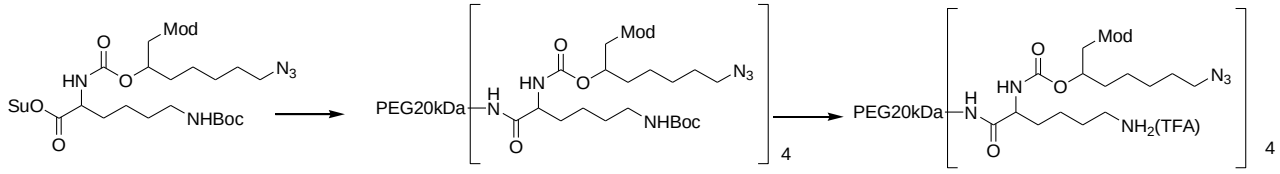
3. Hydrogel prepolymer synthesis



A. PEG_{20kDa}-[NH-MFCO]$_4$ (Prepolymer B). A solution of PEG_{20kDa}-[NH_2•HCl]$_4$ (1700 mg, 0.34 mmol NH_2, 1 equiv) and DIPEA (88 mg, 0.68 mmol, 2 equiv) in MeCN (6 mL) was treated with a solution of MFCO-OPfp [3] (134.5 mg, 0.40 mmol, 1.2 equiv) in MeCN (1 mL). The mixture was kept at room temperature for 15 h when <0.5% residual amines remained by TNBS assay [1]. To cap residual amines the mixture was treated with Ac_2O (34.7 mg, 0.34 mmol, 1 equiv) for 30 min at room temperature, then MTBE (500 mL) was added to precipitate the product. The precipitate was collected by filtration, washed with MTBE (8 x 50 mL), and dried under briefly vacuum to provide PEG_{20kDa}-[NH-MFCO]$_4$ (1.6 g, 91 % yield) as a fluffy white solid. The MFCO content of the weighed product was determined to be 98.5% by consumption of PEG_7-N_3 as determined by DBCO titration [2].



B. (7-azido-1-[($MeOCH_2CH_2$)$_2NSO_2$]-2-heptyloxycarbonyl)-Lys(Boc)-OH. A solution of H-Lys(Boc)-OH (207 mg, 0.84 mmol, 1.05 equiv) in aqueous $NaHCO_3$ (0.5 M, 8 mL) was treated with a solution of (7-azido-1-[($MeOCH_2CH_2$)$_2NSO_2$]-2-heptyloxy)-carbonate-HSC (0.80 mmol, 1.0 equiv) in MeCN (8 mL) and allowed to stand for 18 h. At that time, the linker-HSC was consumed as determined by TLC (R_f ~0.25, ninhydrin) and the MeCN was removed under reduced pressure. The aqueous solution was diluted with water (8 mL), washed with EtOAc (3 x 10 mL), then acidified to pH 3.5 with 1 N HCl upon which an oil precipitated. The product was extracted with EtOAc (4 x 10 mL), then the combined EtOAc extracts were washed with water (3 x 10 mL), brine (10 mL), and then concentrated to dryness. The crude oil product was purified on a silica column (12 g silica/ ~0.6 mmol) using a gradient of 10-50% acetone in hexanes. The product eluted with 50% acetone, to give 260 mg (52% yield) as a clear oil; ESI-MS M+1 = 625.3



C. [7-azido-1-[($MeOCH_2CH_2$)$_2NSO_2$]-2-heptyloxycarbonyl)-Lys(Boc)-NH]$_4$-PEG_{20kDa}. (7-Azido-1-[($MeOCH_2CH_2$)$_2NSO_2$]-2-heptyloxycarbonyl)-Lys(Boc)-OH (230 mg, 0.368 mmol, 1 equiv) was treated with a solution of N-hydroxysuccinimide (NHS) (44.4 mg, 0.386 mmol, 1.05 equiv) in THF (5 mL), and a solution of DCC (79.7 mg, 0.386 mmol, 1.05 equiv) in THF (3 mL) at 4 °C. The resulting mixture was kept for 18 h at 4 °C, filtered through a cotton plug to removed precipitated DCU, then added to a solution of PEG_{20kDa}-[NH_2.HCl]$_4$ (1.60 g, 0.320 mmol (NH_2), 0.83 equiv) and DIPEA (0.112 mL, 83 mg, 0.640 mmol, 2 equiv) in MeCN (12 mL). After 2 h the reaction mixture showed <1% free amines by TNBS assay using a PEG_{20kDa}-[NH_2.HCl]$_4$ standard. The reaction was treated with AC_2O (32.6 mg, 320 mmol, 1 equiv/NH_2) for 1 hour, then concentrated to ~ 5 mL, and treated with MTBE (100 mL). The precipitated product was

collected by filtration, washed with MTBE (5 x 50 mL) then dried under vacuum to give 1.7 g, 95% yield of product as a white solid that was used as is in the following step.

D. [7-azido-1-[$(MeOCH_2CH_2)_2NSO_2$]-2-heptyloxycarbonyl)-Lys(NH_2)-NH]$_4$-PEG_{20kDa} (Prepolymer A). A solution of [7-azido-1-[$(MeOCH_2CH_2)_2NSO_2$]-2-heptyloxycarbonyl)-Lys(Boc)-NH]$_4$-PEG_{20kDa} (1.7g) in 20 mL of 1:1 TFA:DCM was kept for 60 min at room temperature, then concentrated under vacuum to a thick oily residue that was treated with Et_2O (200 mL). The precipitate was recovered by filtration, washed with Et_2O (200 mL) and MTBE (4 x 200 mL) then dried under vacuum to give the product (1.65 g, 97%) as a white solid; the product contained 97% of the expected azide by reaction with DBCO [2].

4. Microsphere preparation. A. Microfluidic production of amino-microspheres. A two-reagent hydrophobic flow focusing microfluidic chip with seven parallel 50 µm drop forming channels (Dolomite, Telos) was used to prepare microspheres. Fluid flow was controlled by a custom-fabricated gas-pressure driven pump, similar in function to the Mitos Pressure Pumps manufactured by Dolomite Microfluidics. The driving pressure is computer-controlled using proportional pressure regulators (Proportion Air, MPV series) to maintain a stable flow rate using a feedback loop from a liquid flow sensor (Sensirion, SLI-0430). Flow control is scalable to deliver liquid from 0.5 mL to multi-liter reservoirs, and produces constant flow rates with ±1% SD. This system was used to deliver the two hydrogel prepolymer solutions as well as the continuous phase. Typical flow rates were 2.1 mL/h for each prepolymer solution and 14 mL/h for the continuous phase. The continuous phase was composed of decane containing 1% w/v Abil-EM90 (Evonik) and 1% w/v PGPR (Danisco). The outlet tube of the device was connected to a fraction collector (Gilson FC203B), and fractions were collected in 10 min intervals. Quality control was performed by photographing the chip at 5x magnification (Fig. S1 A) with a high speed camera (Uni Brain, Fire-I 580b) attached to a microscope (Nikon, EQ-51436) equipped with an automated stage to visualize the seven channels of the chip. Images of each channel were collected every 5 minutes, and fractions containing large particles resulting from device failure were discarded. Generally, device failure occurred after 12- to 20 h of operation due to hydrogel polymerization in a drop-forming channel, but the device could usually be regenerated about four times by washing 15 min with water or 70% ethanol. A single 7-channel device produced 8 mL/hr of water-swollen microspheres, which is scaleable up to ~10-fold using the Telos droplet manufacturing system (Dolomite).

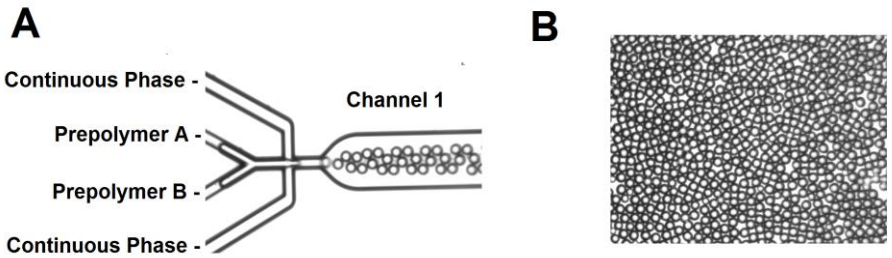


Figure S1. Microfluidic production of hydrogel microspheres. **A.** Micrograph of the flow focusing microfluidic device used to make microspheres. Microspheres are produced at a rate of ~4000/sec/channel. **B.** Hydrogel microspheres (diameter = 42 ± 3 µm) suspended in decane with surfactants.

B. Isolation and sterilization of amino-microspheres. Wash solvents were filtered through 0.2 µm Nylon-66 filters (Tisch, SPEC17984) and microsphere washes were conducted in 50 mL

Teflon® (FEP) centrifuge tubes (Oak Ridge, 3114-0050). After washing, the microspheres were recovered by centrifugation for 5 min at 3000 x g for separation of decane or pentane phases, and 20 min at 3000 x g for separation of aqueous phases.

After production, a 30 mL suspension of microspheres in decane containing surfactant was allowed to polymerize at room temperature for 24 h. The excess decane was aspirated, and the microspheres were partitioned between 15 mL 0.1% w/v aqueous NaN_3 to cap any unreacted MFCO groups and 30 mL pentane. The mixture was agitated for 30 min and the pentane phase was separated by centrifugation. The microsphere suspension was diluted with 30 mL water and washed with 5 x 40 mL portions of pentane. After centrifugation, the excess aqueous phase was removed and the slurry was treated with an equal volume of 50% v/v TFA for 30 min for sterilization [4]. The microspheres were recovered by centrifugation at 1000 x g, and the pellet was neutralized with 150 mL of 0.125 M Na_2HPO_4 to give a suspension with pH ~6.5. After swelling for 18 h the spheres were recovered by centrifugation, then washed with 100 mL of water and 5 x 100 mL of 70% ethanol. The slurry was pelleted at 3000 x g for 30 min. After aspiration of the supernatant, the microsphere slurry was aseptically transferred to a 60 mL syringe (BD No. 309653) that was connected by a Luer coupling to another 60 mL syringe and homogenized by several back-and-forth passages to disperse small clumps. The syringe containing the slurry was used to load individual 10 mL syringes through the Luer coupling that were stored at 4 °C until use.

C. Assay for slurry concentration of cleavable amino-microspheres. Three 0.100 mL portions of amino-microsphere slurry in 70% EtOH were weighed to determine their density (0.87 ± 0.2 g/mL), then each portion was then treated with 0.900 mL of 50 mM NaOH for 18 h at room temperature to cleave crosslinks and form $[H_2N\text{-}Lys(NH_2)\text{-}NH]_4\text{-}PEG_{20kDa}$ monomers. Each sample was assayed for total amine concentration by TNBS assay by diluting 0.030 mL to 0.120 mL with 100 mM borate buffer, pH 9.3, in a microtiter plate and then treating with 0.150 mL of borate buffer containing 0.04% w/v TNBS. The change in absorbance of the TNBS reactions at 420 nm was monitored for 3 h in a plate reader at 25 ^oC then the final absorbance at 420 nM was recorded. Equivalent reactions containing TNBS alone were used for background subtraction and reactions containing 10-, 20- and 40 μM Lys were used for amine concentration standards. The total amine concentration/2 provides the free ε-amine content in the 30 μL of gel digest assayed.

5. Microsphere reactor and washer. Fig. S2 shows an aluminum fixture fabricated to hold calibrated 10 mL syringes (BD 309604) in a centrifuge rotor for standard 50 mL conical centrifuge tubes. The tared syringes are loaded with microspheres by syringe-to-syringe transfer through a Luer lock coupling from syringe-loaded stock slurries. For solvent additions, the solvent (up to 3-volumes of microsphere slurry) is introduced through a sterile filter into the Luer coupling to the reaction syringe, and the syringe is then plugged with a female Luer lock cap (BD No. 408531); after reaction or solvent addition the vessel is centrifuged in an inverted position (plunger down) at 3,000 x g until the microspheres sediment as a packed slurry against the plunger (ca. 5 min for organic, 20 min for aqueous solvents); the volume of microsphere slurry is measured using the syringe calibrations. For solvent removal, the centrifuged vessel is uncapped, fitted with a needle containing a polypropylene frit (Scientific Commodities; GP-272) within its female Luer lock fitting, and excess solvent dispelled from the inverted syringe by displacement with the plunger; the total weight minus the weight of the tared vessel is used to determine the slurry weight. At any time, samples of the slurry or supernatant can be expelled through the Luer port for analysis. For sequential reactions, the material made in the first reaction is carried on to the second step either within the same vessel, or after syringe-to-syringe transfer into multiple similar reaction vessels. Reaction volumes up to 8 mL can be

performed in a single syringe, after which ~2 mL portions are transferred syringe-to-syringe into individual 10 mL syringes for washing. Three volumes of wash dilute the packed slurry solvent to ~0.4%.

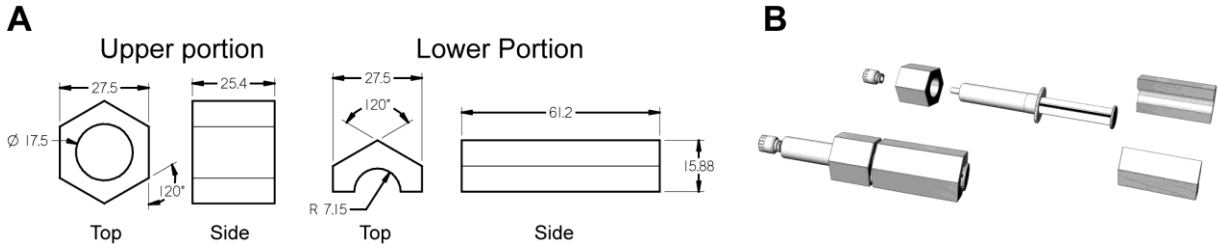


Figure S2. Fixture used for centrifugation of 10 mL Syringes. **A.** Scale drawing of the parts. Dimensions are in millimeters and degrees. **B.** A 3D model of the fixture and syringe showing assembled and exploded views.

6. MFCO derivatization of amino-microspheres. The reaction is performed in the syringe reaction vessel as follows. For each **4** mL of a packed suspension of amino-microspheres in MeCN containing 2 µmol amine/mL gel slurry are added 32 µmol DIPEA (4 equivalents) in 1 mL MeCN, and 9.6 µmol (1.2 equivalents) of MFCO-PFP in 1 mL MeCN. After 1 h rocking at ambient temperature, a small amount (~50 µL) of microspheres is expelled from the syringe outlet and treated with 0.5 mL of 0.04% w/v TNBS in 0.1 M sodium borate, pH ~9.3 [1] for 30 min; complete reaction is indicated by a microsphere color matching the TNBS solution compared to starting amino-microspheres which stain an intense orange. After reaction, the microspheres are capped by the addition of 8 µmol (1 equivalent) of Ac_2O in 1 mL MeCN for 10 min. After removal of the supernatant, ~2 mL of the microspheres is transferred to a second 10 mL syringe, each slurry is washed with 4 x 3 volumes MeCN per packed slurry volume and the slurries combined.

7. Exenatide-microspheres. The SPAAC reaction is performed in the above syringe reaction vessel as follows.To a suspension of 2.4 g of a slurry of MFCO-derivatized microsphere (11.2 µmol MFCO) in 30% MeCN in a 10 mL syringe was added a solution of 46 mg (10.4 µmol) of N^{α}-[1-(methylsulfonyl)-7-azido-2-heptyloxycarbonyl]-exenatide in 2 mL 30% MeCN. The mixture was slowly rotated until the OD_{280} of an aliquot was constant at ~24 hr. About 50% of the slurry was transferred to a second syringe, and both samples were washed with 4 x 2 mL of 30% MeCN and then 5 x 5 mL of 10 mM NaP_i, 0.04% Tween 20, pH 6.2. The exenatide-loaded microspheres were then syringe-to-syringe transfered to several 1.0 mL dosing syringes. The total loading of the microsphere was 1.9 µmol exenatide gm^{-1} of slurry as determined by the total peptide released at pH 8.4 (below).

8. Release kinetics and reverse gelation time. Kinetics of β-elimination were determined in duplicate under accelerated release conditions using 80 mg of the microsphere-exenatide slurry in 200 µL of 100 mM Bicine, pH 8.4 at 37 °C. Samples were centrifuged at 20,000 x g in a microfuge tube and A_{280} of the supernatant was measured on a NanoDrop 2000 spectrophotometer (ThermoScientific) at intervals. The release rate was calculated by fitting the released A_{280} vs time to the first-order rate equation. Knowing that the β-elimination is first-order in hydroxide ion [1] rates were calculated at pH 7.4 as $k_{pH\ 7.4} = k_{pH} \times 10^{(7.4-pH)}$.

A trace amount of 6-azidohexanoyl-aminoacetamido fluorescein [1] was attached to remaining MFCO groups of 150 mg of exenatide microspheres, which was then placed in a ~2.5 cm x 0.5

cm “tea-bag” prepared from heat-sealed 335-mesh nylon (37 μm pore; McMaster-Carr, 9318T24). The bag was placed into the top compartment of a cuvette fitted with a magnetic stirrer and a separating metal mesh support to hold the bag in the reaction solution and above the light path [5]. The cuvette contained 2.5 mL of stirred 100 mM Na borate, pH 9.4, 37 °C, and A_{495} was continuously monitored until all microspheres dissolved; t_{RG} at pH 7.4 was calculated as $t_{RG,pH\ 7.4} = t_{RG,pH} \times 10^{(7.4-pH)}$.

9. Pharmacokinetics of microsphere-exenatide conjugates in the rat. The contents of tared 1 mL dosing syringes containing the exenatide-microsphere slurry were administered through a 27 gauge needle s.c. in the flank of cannulated male Sprague Dawley rats, ~350 g. Each syringe contained 0.45 or 0.98 μmol exenatide at 1.9 μmol exenatide g^{-1} slurry. The syringes were weighed prior to and after dosing to verify the mass delivered to each rat. Blood samples (300 μL) were drawn at 0, 1, 2, 4, 8, 24, 48, 72, 120, 168, 240, 336, 408, 504, 576, 672 hours, and sera was frozen at -80 °C until analysis.

Exenatide concentrations in sera were measured by ELISA according to the manufacturer’s protocol (Peninsula Laboratories Inc., #S-1311). Frozen serum samples were thawed on ice and diluted between 5 and 20-fold in the provided rat serum. The standard exenatide showed an IC_{50} = 0.22 nM (reported 0.19 nM). Data replicates were averaged and fitted to appropriate pharmacokinetic models using Nelder-Mead downhill simplex [6]. Accuracies of fits were determined as ±SE of the best-fits of data to curves used to determine kinetic parameters.

Supplementary Information References

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