Peptide hydrogels as slow-release formulations of enzyme therapeutics: Case study of asparaginase-loaded hydrogels

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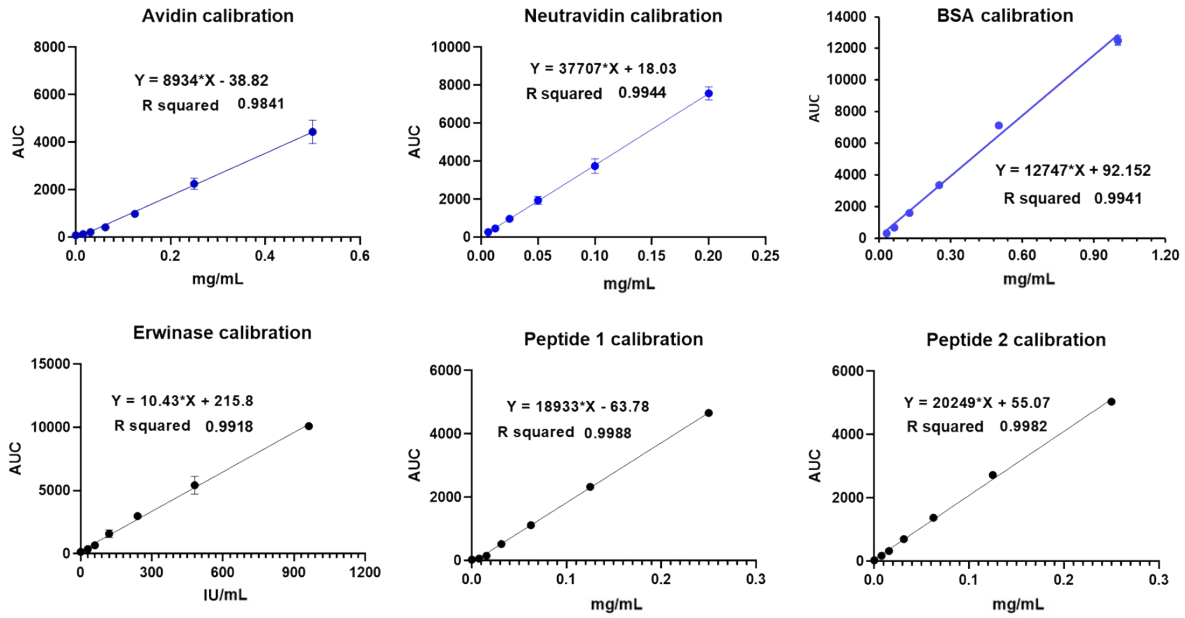
Experimental Section

Reversed-phase high-performance liquid chromatography (RP-HPLC)

The samples from the *in vitro* release experiments were analyzed via reversed phase high performance liquid chromatography (RP-HPLC) using an Agilent 1260 series instrument equipped with a Jupitor C18 300Å column (250 x 4.60 mm, 5 µM particle size at 35°C) (from Phenomenex) connected to a DAD using a flow rate of 1.5 mL/min with the following solvent system: (A) MQ water + 0.1% TFA and (B) MeCN. The column was flushed for 2 minutes with 100% A, then a gradient from 0 to 100% B in 20 min was used, followed by 2 minutes flush of 100% B, and 1 minute with 100% A. All release samples (in PBS buffer) were diluted with equal volume of H_2O/MeCN (50:50 v/v) and stored at -20 °C until the time of measurement. Absorption chromatogram at 214 nm was used for analysis. Calibration curves of protein cargos as bellows:



HRP-streptavidin release study

HRP-Streptavidin (1.25 mg/mL stock solution from Thermal Fisher Scientific) was loaded to hydrogels of **2** (H-FQFQFK-NH_2) by mixing 50 µL of 10 µg/mL enzyme in PBS 2X with 50 µL of hydrogelator **2** in water (4 %w/v) in an Eppendorf tube to afford a transparent hydrogel at final concentration of 5 µg/mL protein and 2 %w/v peptide (triplicates). The tubes were kept at 4 °C for aging. The next day, 500 µL of PBS 1X was carefully added on top of the hydrogels to start the release experiment. At the specific time point, 10 µL of the release buffer was collected for enzymatic activity measurement. For the activity measurement, the release sample was diluted 500 times in PBS buffer containing 0.5% BSA. In each well of the 96 well plate, 50 µL of the reaction mixture containing 3,3',5,5'-tetramethylbenzidine (TMB) substrate and hydrogen peroxide was added to 50 µL of the diluted release samples. The plate was incubated for 10 min at room temperature, after which 50 µL of the sulfuric acid 0.16M was added to stop the reaction. The absorbance was read at 450 nm within 15 min.

Microbial transglutaminase (mTG) release study

mTG powder from supplier (Bindly) was reconstituted in PBS 2X buffer (200 mg/600 µL). The slightly slurry solution was then carefully filtered through 0.22 µm filter, repeated twice. The transglutaminase activity in the stock solution was determined using microbial transglutaminase assay kit (Zedira, no. Z009). The enzyme was loaded to hydrogels of **1** (H-FEFQFK-NH_2) or **2** (H-FQFQFK-NH_2) by mixing 50 µL of the stock solution with 50 µL of hydrogelator in water (4 %w/v) in an Eppendorf tube to afford transparent hydrogels (duplicates). Hydrogels formulated without enzyme were used as blank controls

while enzyme in PBS was used as positive control. The tubes were kept at 4 °C for aging for 2 hours. Next, 400 µL of PBS 1X was carefully added on top of the hydrogels to start the release experiment. At the specific time point, 10 µL of the release buffer was collected and added to each well of a 96 well plate. The enzymatic activity measurement was done using Zedira microbial transglutaminase assay kit. To each well of the 96 well plate containing the release samples, 140 µL of the reaction mixture containing N-Benzyloxycarbonyl-L-Glutaminylglycine (Z-Gln-Gly) and hydroxylamine (140 µL) (priorly prewarmed to 37°C). The plate was incubated at 37°C for 10 minutes, after which a stop solution containing iron (III) was added to each well (150 µL/well). In the presence of mTG, hydroxylamine is incorporated into Z-Gln-Gly to form Z-glutamyl(γ-hydroxamate)-glycine which develops a colored complex with iron (III). The release samples from hydrogels without enzymes were used for blank reading. The absorbance signal was detected at 525 nm with DropSense 96 (Trinean) spectrophotometer. Signals after blank subtraction were normalized using reading of positive control (enzyme in PBS).

Plasma L-asparaginase activity determination

Measuring asparaginase activity in low range (5-100 IU/L): Standards (5, 10, 20, 30, 50, 75, 100 IU/L) and controls (10, 50, 100 IU/L) were generated through serial dilutions of Erwinase in TRIS/BSA 5%-buffer (pH 7.3) and measured as for samples of interest. A volume of 20 µL sample was added to a well of 96-well plate (VWR, 732-2661). A volume of 180 µL of a 2 mM solution of AHA substrate was added to the sample and incubated 30 minutes at 37 °C. The reaction was stopped by adding 60 µL of 24,5% (w/v) trichloroacetic acid solution (Alkemi A5055). After a 5 minutes centrifugation step at room temperature, 830xg, 50 µL was added to a well of a 96-well plate (Sarstedt 82.1581). A volume of 200 µL Oxine reagent was added and incubated at 95 °C for 1 minute. Oxine reagent for one full 96-well plate consisted of 18 mL 1 M Na_2CO_3 (Sigma Aldrich 31432) and 6 mL 2% (w/v) 8-hydroxyquinoline (Merck 820261). The plate was allowed to cool down for 10 minutes and subsequently read at 690 nm using a SpectraMax M3 (Molecular Devices) spectrophotometer. SoftMax Pro software (v6.2.2; Molecular Devices, LLC) was used for instruments' control and data acquisition. Standard curve fitting was done using a quadratic fit model and was only accepted with an $R2 > 0.9$. Five out of seven standards and seven out of nine controls should fall within the expected CV value ± 20%.

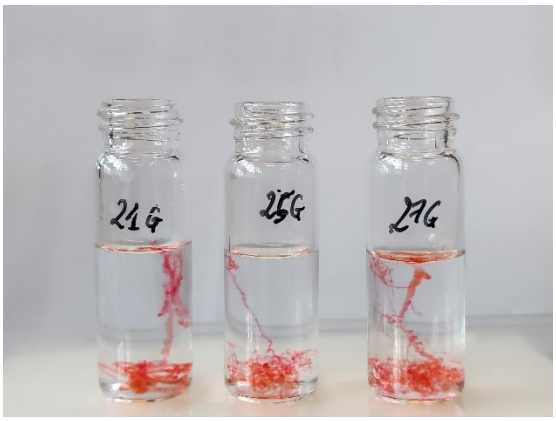
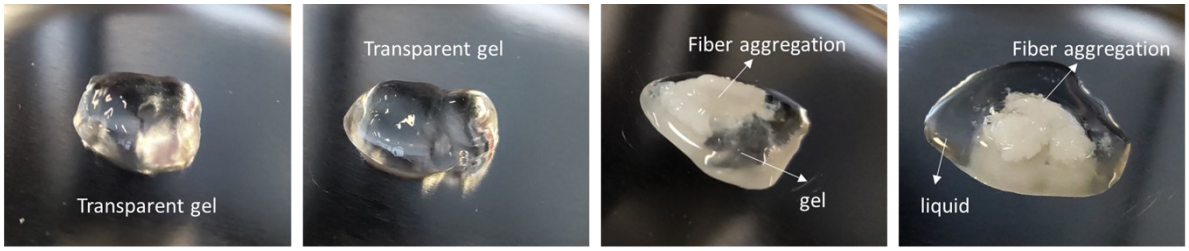


Figure S1. Injectability of hydrogel with different needle sizes. Hydrogel of **2** (H-FQFQFK-NH_2) loaded with red dye for better visualization after being pushed out from different needle sizes (21G – left, 25G – middle, and 27G – right). In all cases, the hydrogels instantly re-adopted their gel-like behaviors. The bottles contain PBS buffer (pH7.4).



BSA loading	0 mg/mL	2.5 mg/mL	25 mg/mL	50 mg/mL
Peptide 1	2% w/v	2% w/v	2% w/v	2% w/v

Figure S2. Visual observation of BSA-loaded hydrogel of 1 (H-FEFQFK-NH_2) at different loading concentrations. The hydrogels were prepared the day before in syringe, left for aging at room temperature overnight. The images are hydrogels after being pushed out from the syringe (without using needle).

Figure S3. Visual observation of hydrogel of 1 and 2 loaded with different model cargoes and the corresponding gel residue after 48 hours post *in vitro* release. Transparent hydrogels were obtained for all model cargos at the tested concentration (1 mg/ml). Hydrogels of peptide 2 formulated with model cargos remained stable with little erosion in PBS release buffer while the hydrogel of peptide 2 significantly eroded after 48-hour post release (small amounts of remaining gel residues are marked with red circles).

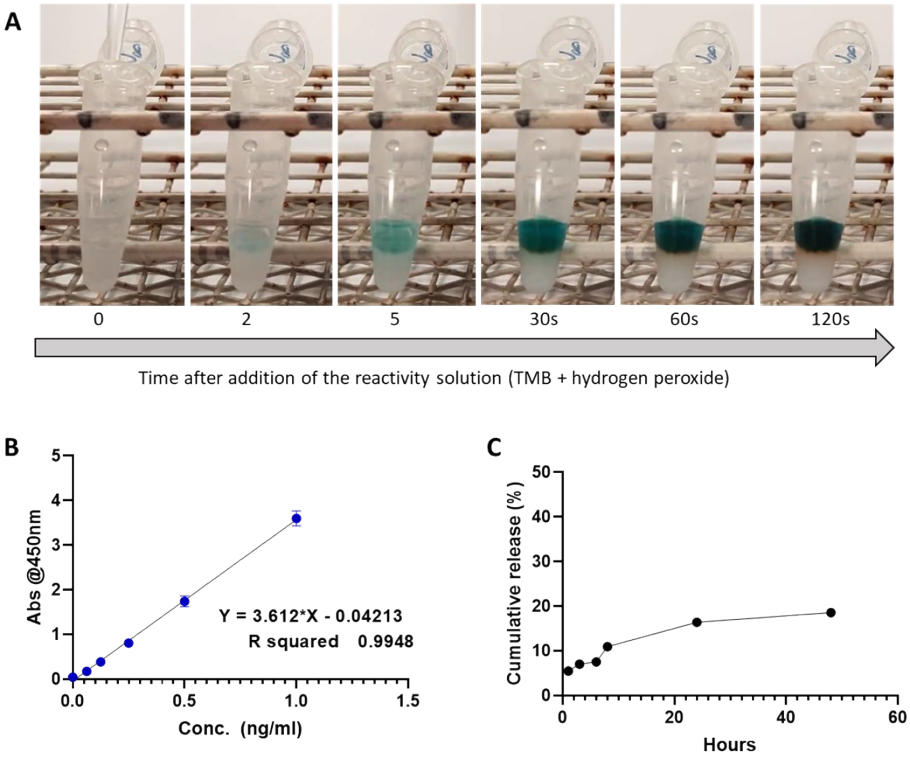


Figure S4. Real-time activity and release of HRP enzyme from the hydrogel. **A)** Time-lapse photo shots of hydrogels of **2** (H-FQFQFK-NH_2) loaded with HRP-streptavidin after addition of the reactivity mixture containing TMB substrate and hydrogen peroxide directly on top of the hydrogel. **B**) HRP-streptavidin calibration curve and **C**) The cumulative release curve of HRP-streptavidin from the hydrogel of **2** assuming that no activity was lost during the experiment. Mean values ± SD are represented (n = 3).

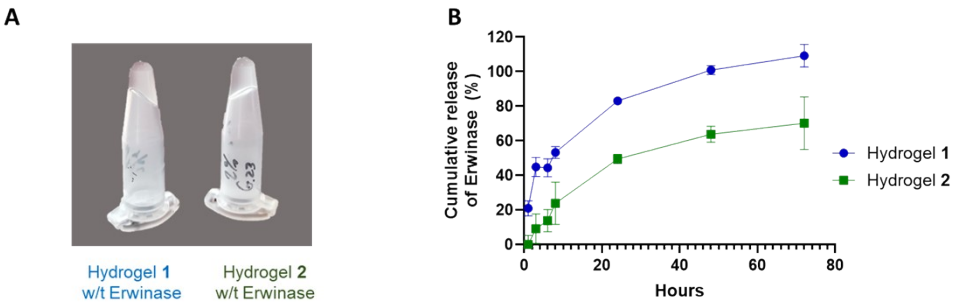


Figure S5. Sustained-release hydrogel formulations of Erwinase. A) Inverted tubes of Erwinase-loaded hydrogel **1** (left) and hydrogel **2** (right) (2 %w/v). **B**) *In vitro* cumulative release of Erwinase from enzyme-loaded hydrogel **1** (blue line, filled circle) and **2** (green line, filled square).

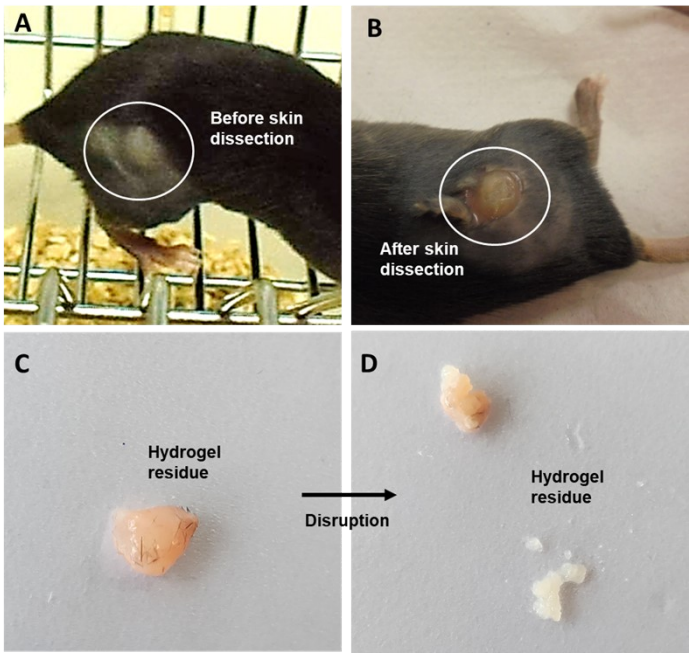


Figure S6. Hydrogel residue 7 days post injection. Depot of hydrogel of **2** (H-FQFQFK-NH_2) loaded with L-asparaginase 7 days post subcutaneous injection, before skin dissection (**A**) and after skin dissection (**B**) to examine the hydrogel residue (**C, D**). Meanwhile, no visible depot was observed in the case of asparaginase-loaded hydrogel of **1** after 7 days post injection.

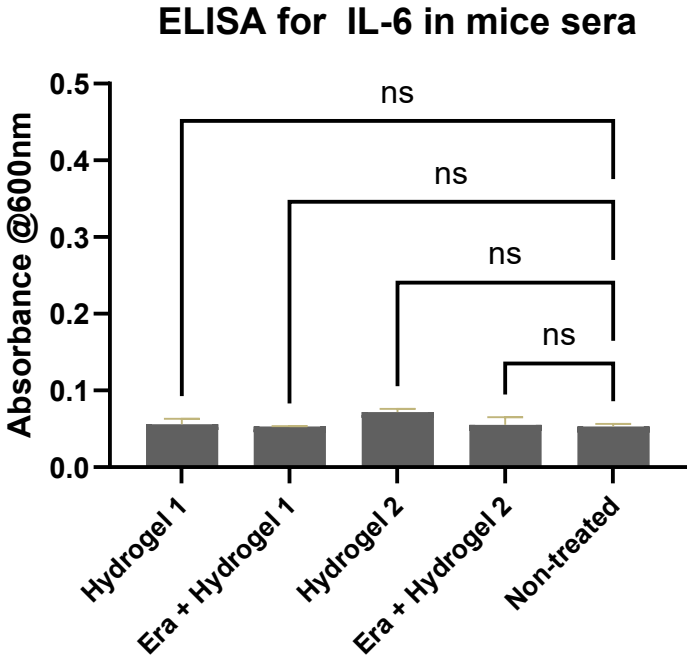


Figure S7. Measurement of the inflammatory cytokine (IL-6) signal in mice sera via ELISA. Sera of mice 2 days post injection with hydrogel **1** and **2** with and without Erwinase (Era) as compared to the control without treatment. No statistically significant elevation in IL-6 was observed in all cases as compared to the non-treated control (one-way ANOVA, ns $p > 0.05$). Mean values ± SD are represented (n = 2).

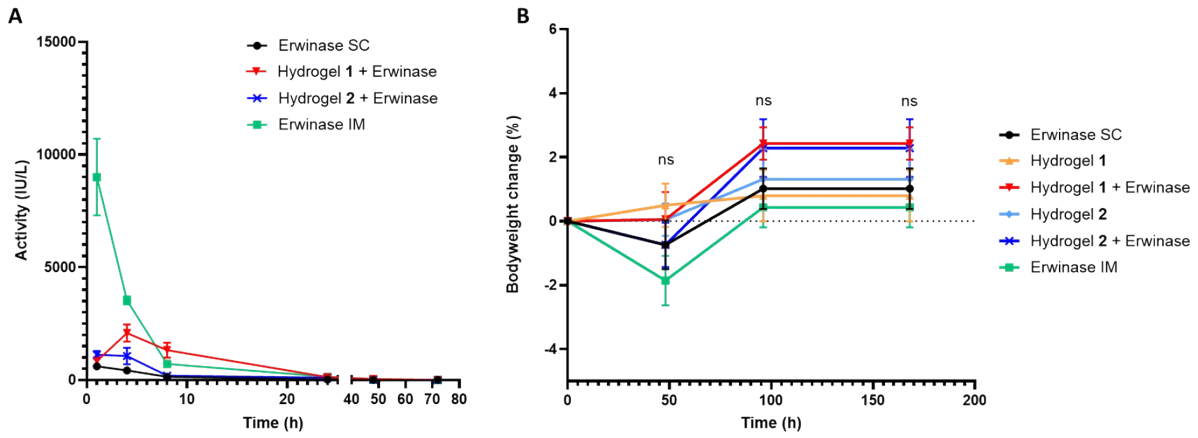


Figure S8. ***In vivo*** **pharmacokinetics of Erwinase – hydrogel complexes, with intramuscular Erwinase injection included as control.** (**A**) ASNase activity measurements (IU/L) in blood plasma samples of mice obtained at 4, 8, 24, 48 and 72 hours after injection of 5000 IU/kg Erwinase, either formulated in PBS (as in Erwinase SC and Erwinase IM, SC = subcutaneous, IM = intramuscular), or encapsulated in hydrogel **1** or **2**. Mean values ± SEM are represented (n = 6). (**B**) Bodyweight fluctuations (%) of mice as the measure of toxicity. Mean values ± SEM are represented (n = 12). No significant difference in bodyweight changes was observed when compared between groups (two-way ANOVA, ns p> 0.05).

Table S1. Average asparaginase activity (IU/L) of the serum samples from the PK study (n=6)

	1h	4h	8h	24h	48h	72h
Hydrogel controls	< 5	< 5	< 5	< 5	< 5	< 5
Erwinase	502	246	142	69	< 5	< 5
Hydrogel 1 + Erwinase	785	2601	1329	159	45	< 5
Hydrogel 2 + Erwinase	1249	977	196	131	48	< 5

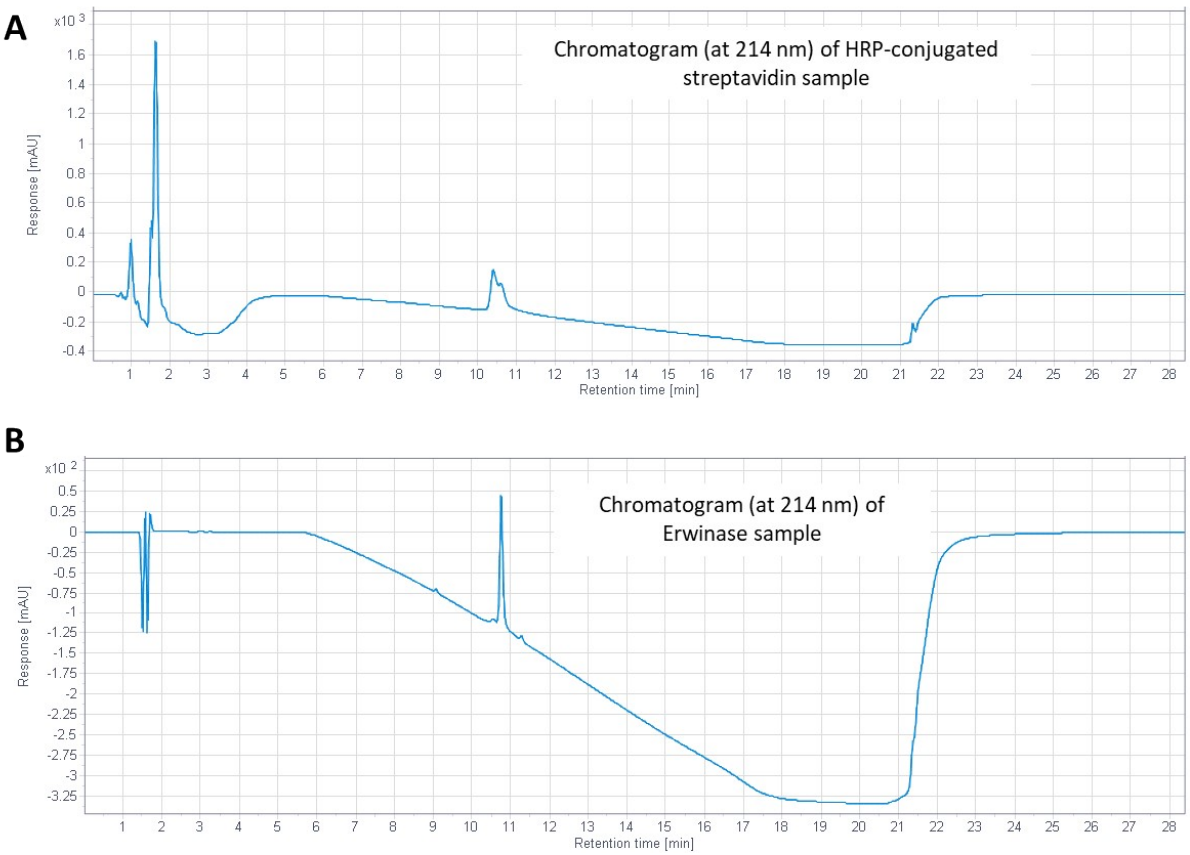


Figure S9. Homogeneity of the enzyme cargo samples via HPLC analysis. A) HRP-conjugated streptavidin sample (0.025 mg/mL) eluted on a Nucleodur C18 300A column. The overlapping peaks at 10.5 min indicate that different conjugate species are present in the protein samples and B) Erwinase sample (0.01 mg/mL) eluted on a Jupiter C18 300A column.