Supporting Information

Enhanced Catalysis of Oxime-Based Bioconjugations by Substituted Anilines

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Calculation of Reaction Rates: The reaction between glyoxylyl-derivatized T3 protein **2** and aminooxy-PEG **3** is a bimolecular reaction following second order rate kinetics (assumption: $k_{forward} >> k_{reverse}$). The rate of formation of the oxime product **4** is related to the concentration of reactants according to equation (1).

$$\frac{d[\mathbf{4}]}{dt} = k_{obs}[2][3] \qquad (1)$$

Integration of (1) leads to equation (2), which relates the concentration of **4** to time and to the concentrations of **2** and **3** at t = 0 ($\mathbf{2_0}$ and $\mathbf{3_0}$).

$$k_{obs}t = \left\{\frac{1}{[\mathbf{3}]_0 - [\mathbf{2}]_0}\right\} \times \ln\left\{\frac{[\mathbf{2}]_0([\mathbf{3}]_0 - [\mathbf{4}])}{([\mathbf{2}]_0 - [\mathbf{4}])[\mathbf{3}]_0}\right\} \qquad (2)$$

For all calculated rate constants in this work (Tables 1, 2 and S1), $\mathbf{2_0}$ = 86 μM and $\mathbf{3_0}$ = 455 μM (concentration $\mathbf{2_0}$ assumes 95% conversion of 91 μM T3 protein into its α-N-glyoxylyl derivative following oxidation by $NaIO_4$). For this set of initial reactant concentrations, $\mathbf{3_0}$ = 5.3 x $\mathbf{2_0}$. Substitution of this into (2) leads to equation (3), which can be rearranged to give equation (4), a function giving the concentration of product **4** as a function of time.

$$k_{obs}t = \left\{\frac{1}{4.3[\mathbf{2}]_0}\right\} \times \ln\left\{\frac{5.3[\mathbf{2}]_0^2 - [\mathbf{2}]_0[\mathbf{4}]}{5.3[\mathbf{2}]_0^2 - 5.3[\mathbf{2}]_0[\mathbf{4}]}\right\} \qquad (3)$$

$$[\mathbf{4}] = \frac{[\mathbf{2}]_0(e^{4.3[\mathbf{2}]_0 k_{obs}t} - 1)}{e^{4.3[\mathbf{2}]_0 k_{obs}t} - 0.19} \qquad (4)$$

Nonlinear regression analysis was used to determine k_{obs}, by fitting experimentally determined values of [**4**], as a function of time, to equation (4) using Kaleidagraph software. Error values for k_{obs} were those reported by the software.

Table S1: Rates of formation of oxime ligation product at pH 7 in the presence of aniline or its derivatives.

Catalyst	**Rate** k_{obs} **(10^{-3} $M^{-1}s^{-1}$)**	$k_{obs(catalyzed)}$/ $k_{obs(uncatalyzed)}$
No catalyst	7.5 ± 0.16	N/A
Aniline	20 ± 0.39	2.7
p-PDA	330 ± 2.5	44
2-Me-*p*-PDA	380 ± 26	51
N-Me-*p*-PDA	500 ± 14	67
p-AP	220 ± 6.0	29
p-MeOAn	150 ± 2.0	20
5-MeO-AA	240 ± 4.6	32

Calculation of second order rate constants (k_{obs}) for formation of T3-PEG conjugate by oxime ligation at pH 7, in the presence or absence of different catalysts. The reaction was performed at a protein concentration of 1 mg/mL (91 μM) and a PEG concentration of 455 μM at room temperature. Product formation was analyzed by HP IEC, and data were fitted to a second order rate equation.

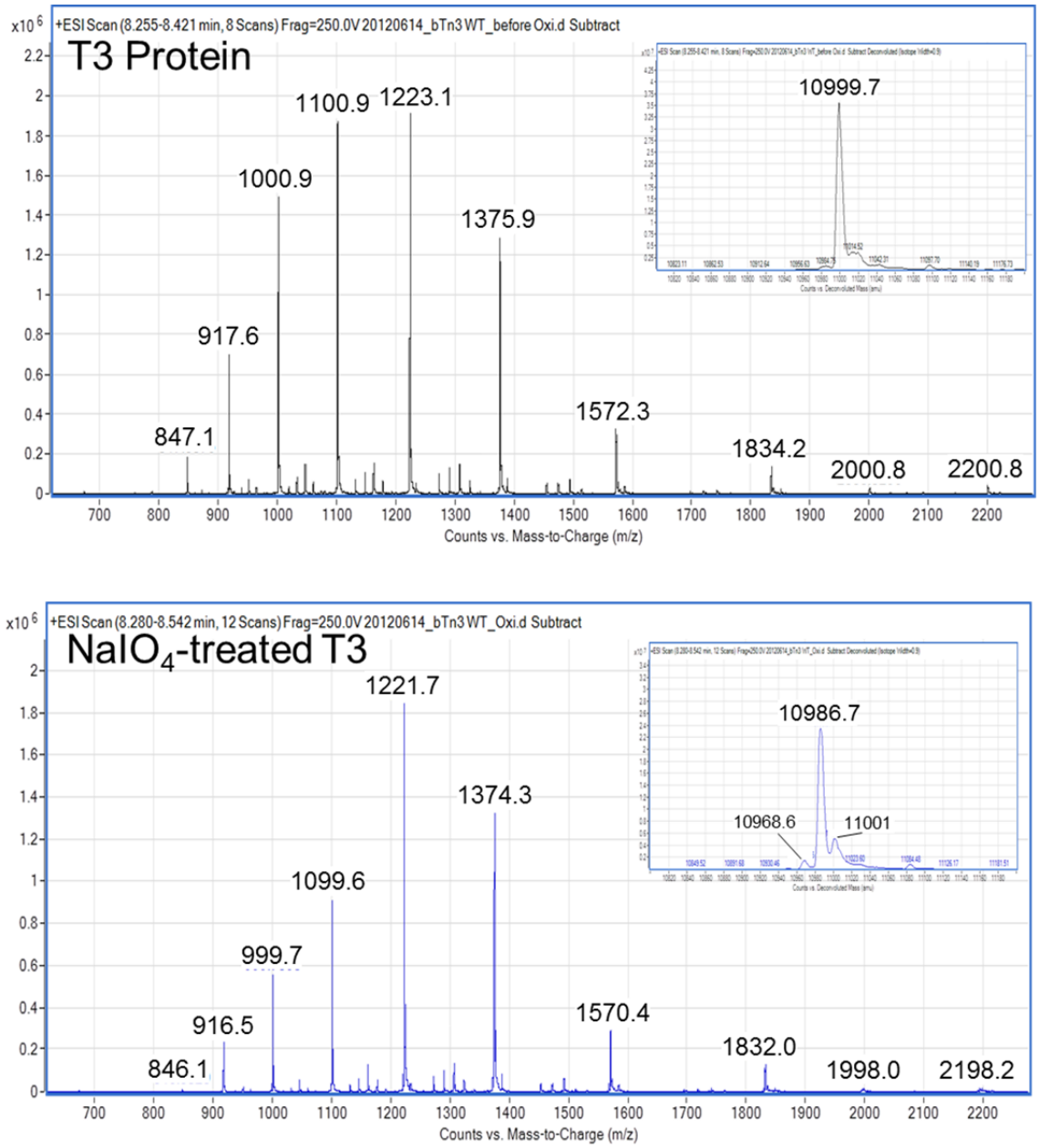

Figure S1: Electrospray mass spectra of T3 protein **1**, and periodate-oxidized T3 protein **2**. Samples were analyzed on an Agilent 6520 Q-TOF LC/MS instrument. Insets show the deconvoluted spectra generated from the corresponding raw data. The experimentally determined masses of T3 protein and its α-N-glyoxylyl derivative (hydrated species) are in good agreement with the calculated masses of 10999.1 Da and 10986.1 Da (average isotope composition). Minor species in the periodate-treated sample correspond to the unhydrated form of the α-N-glyoxylyl moiety (10968.6 Da experimental), and residual unoxidized T3 protein (11001 Da experimental)

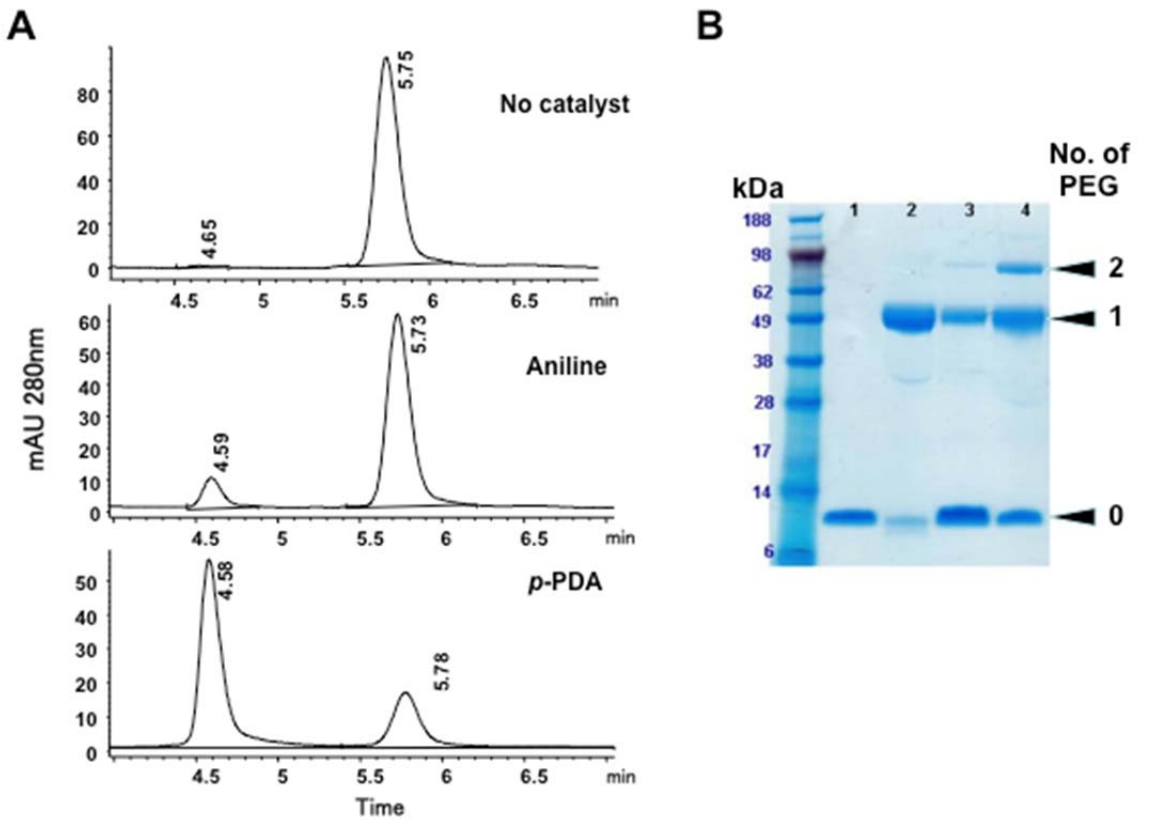

Figure S2: Analysis of PEGylation reaction mixtures. (A) Chomatographic quantitation of T3 PEGylation yields by HP ion exchange chromatography. Under conditions used, α-N-glyoxylyl T3 protein and its oxime PEGylated derivative elute as well resolved peaks at retention times of 5.7 and 4.6 minutes. Integration of these peaks in the 280 nm trace was used to quantify reaction yields. The example shown is from a reaction performed at ambient temperature, pH 6 and quenched after 37 minutes. (B) SDS-PAGE analysis of T3 protein PEGylation. Lane 1: unreacted T3 protein. Lane 2: Site-specific PEGylation of glyoxylyl T3 protein with aminooxy-PEG20 in the presence of 10 mM *p*-phenylenediamine (5:1 aminooxy-PEG:glyoxylyl T3). Lanes 3 and 4: Amine-directed T3 protein PEGylation by α-succinimidyloxyglutaryl-ω-methoxy-PEG (NHS-PEG20: 20 kDa; SUNBRIGHT® ME-200GS, NOF America). Oxime-based PEGylation (lane 2) was carried out with 91 μM periodate-oxidized T3 protein and 455 μM aminooxy-PEG20 in the presence of 10 mM *p*-PDA. Amide-based PEGylation was carried out with 91μM T3 protein and either 455 μM (lane 3) or 910 μM (lane 4) NHS-PEG20, in 100 mM sodium phosphate buffer, pH 7.0. All reactions were incubated for 4 hours at ambient temperature prior to analysis by SDS-PAGE. The random amine-directed PEG conjugation (lanes 3 and 4) was included to contrast the difference in reaction product profiles obtained between this chemistry and catalyzed oxime ligation, and to highlight where mono- and diPEGylated T3 conjugates migrate on the SDS-PAGE gel.

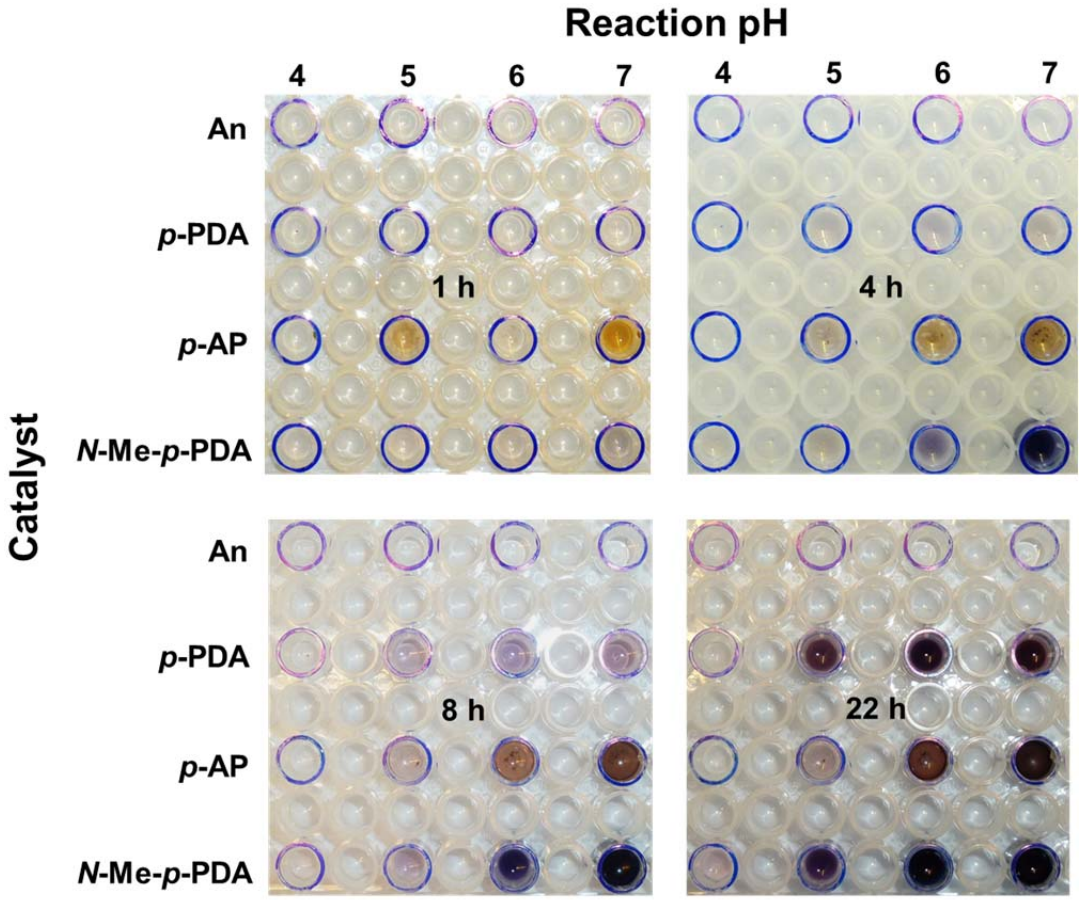

Figure S3: Formation of colored catalyst byproducts in oxime ligations. To illustrate differences in the formation of colored redox products during ligations with *p*-phenylenediamine, *p*-aminophenol and *N*-methyl-*p*-phenylenediamine, oxime ligation reactions were set up in a microtitre plate with 91 μM periodate-oxidized T3, 455 μM aminooxy-PEG and 10 mM catalyst. The plate was covered with foil and left at ambient temperature. Color photographs of the plate were taken at different time points after initiating the reaction (1, 4, 8 and 22 hours).

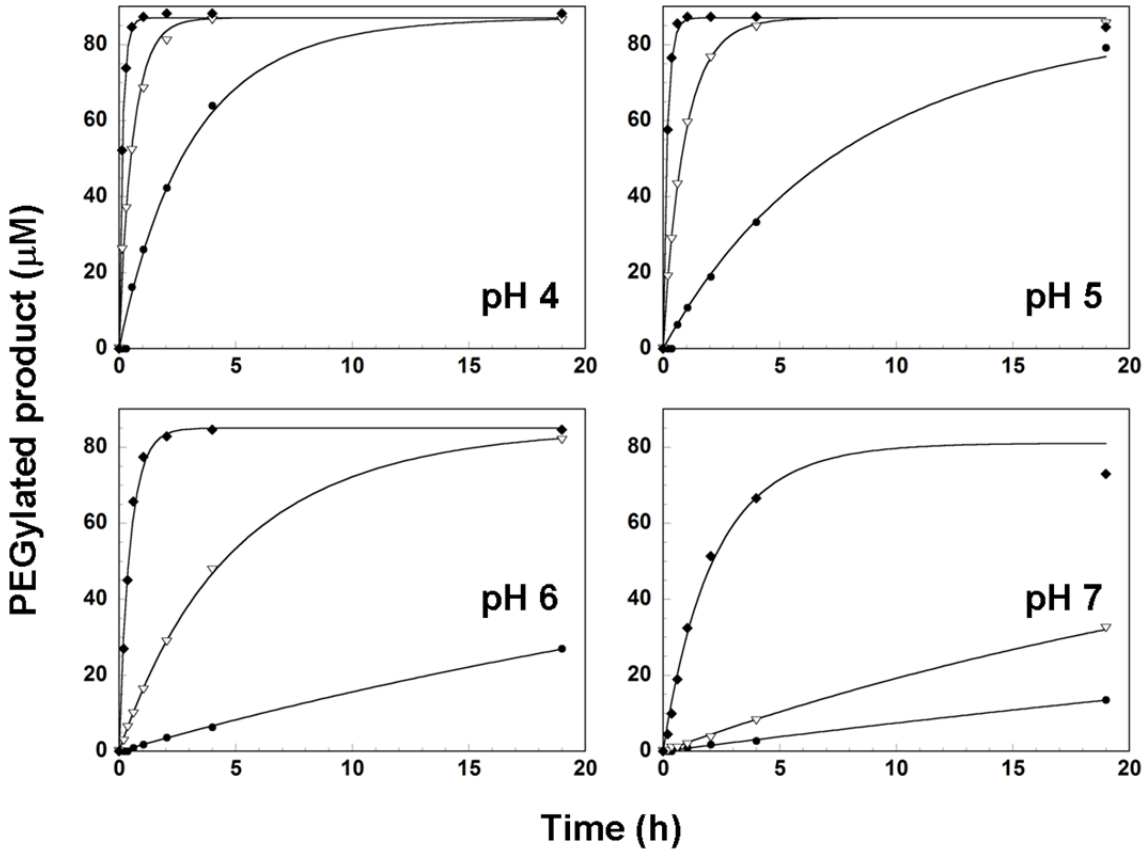

Figure S4: Rate of formation of oxime ligated T3-PEG product at ambient temperature as a function of catalyst and pH. Reactions were with a protein concentration of 1 mg/mL (91 μM) and a PEG concentration of 455 μM in the absence of catalyst (filled circles) or in the presence of aniline (empty triangles) or *p*-phenylenediamine (filled diamonds).