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Studies of the amidation of porphyrin-NHS esters in dilute aqueous solution†

Kittipan Siwawannapong,[a] Zhiyuan Wu,[b] Quang-Thien Ngo[a] and Jonathan S. Lindsey*[a]

Effective bioconjugation of porphyrins is desirable for diverse applications in the life sciences. Two *trans*-AB-porphyrins equipped with a 2,6-bis[$HO_2C(CH_2CH_2O)_7$]phenyl group and the NHS ester of 4-benzoic acid (**P3-NHS**) or 4-phenylpropanoic acid (**P4-NHS**) were examined for the kinetics and yields of hydrolysis and amidation (with a short amino-PEG reagent, $H_2N–(CH_2CH_2O)_4–Me$). The reactions were carried out by combining porphyrin-NHS esters in DMSO (containing 0.05% formic acid) with aqueous sodium carbonate buffer (50 mM) in 1 : 9 ratio and use of controlled conditions (concentration, pH, temperature) followed by HPLC analysis with absorption and mass spectrometric detection. The general conditions employed dilute solution (porphyrin-NHS ester at 1.0 mM and $H_2N–(CH_2CH_2O)_4–Me$ at 2.0 mM) at room temperature. For **P3-NHS** (1.0 mM), the $t_{1/2}$ for hydrolysis at room temperature was 210, 180, or 125 min at pH 8.0, 8.5, or 9.0, respectively, *versus* amidation with $t_{1/2}$ of 80, 20, or 10 min and yield of amide of 80–85%. For **P4-NHS** (1.0 mM), the rates were faster for hydrolysis ($t_{1/2}$ = 190, 130, or 110 min) and amidation ($t_{1/2}$ = 25, 10, or 5 min), with yield of amide of 87–92%. The yield of amide as a function of concentration of NHS ester (with 2 equivalents of $H_2N–(CH_2CH_2O)_4–Me$) was 88% (1 mM), 74% (0.316 mM), and 56% (0.1 mM) for **P3-NHS**, and 97% (1 mM), 89% (0.316 mM), and 73% (0.1 mM) for **P4-NHS**. In both cases, the yield of amide product was slightly greater at room temperature than 37 °C. Both **P3-NHS** and **P4-NHS** were stable in frozen DMSO solutions (−20 °C). The studies establish a quantitative basis for carrying out bioconjugations of water-soluble porphyrins in dilute aqueous media without a large excess of porphyrin-NHS ester or amine. A summary of other quantitative studies of NHS-ester bioconjugations provides valuable context.

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Introduction

The ability to conjugate porphyrins to biomolecules and related entities is valuable for a range of studies in the life sciences. The reaction of NHS esters with amines is a core pillar in the field of bioconjugation. Such reactions are often carried out with a large excess of one or the other reactant, the NHS ester or the amine.

The prior paper[1] concerns the design and synthesis of a set of *trans*-AB-porphyrins each bearing a single bioconjugatable group and a single water-solubilizing group. Two porphyrins wherein the bioconjugatable group is an NHS ester have been advanced for studies described herein. Here, a central concern is the use of dilute solutions of the porphyrin-NHS ester and the amine without a large excess of either reactant. The use of excess NHS ester is attractive to overcome limitations due to competitive hydrolysis in aqueous solution. Counterbalancing that approach is the often-valuable nature of the component (ligand, dye, fluorophore, complex) that bears the NHS ester. On the other hand, many bioconjugation reactions entail use of a protein, which contains numerous amines (of a range of steric accessibility and hence intrinsic reactivity), whereupon the efficiency of bioconjugation may be hard to gauge. The commonplace nature of bioconjugations with NHS esters in bioorganic chemistry is marked by comparatively few quantitative studies of reactions with similar concentrations of NHS-ester and amine in dilute aqueous solution. Knowledge of the kinetics of bioconjugation is essential for effective use, and also for the rational design of candidate drugs in kinetic target-guided syntheses.[2]

In this paper, the two porphyrin-NHS esters have been evaluated for (1) the kinetics of hydrolysis in aqueous solution as a function of pH (8.0, 8.5, 9.0), and (2) the kinetics of amidation with a short amino-PEG reagent as a function of pH (8.0, 8.5, 9.0), concentration (1.0 mM, 0.316 mM, 0.10 mM), and temperature (room temperature, 37 °C). The kinetic studies

[a] *Department of Chemistry, North Carolina State University, Raleigh, NC 27695, USA. E-mail: jlindsey@ncsu.edu*
[b] *Oncurie, Inc., Raleigh, NC 27608, USA*

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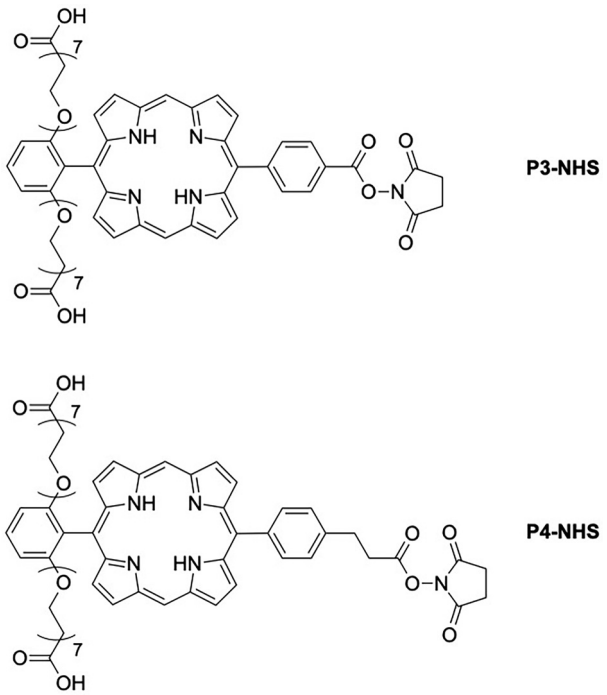

Chart 1 Porphyrin-NHS esters.

have been evaluated by HPLC with mass spectrometry (MS) for species identification and absorption spectroscopy (Abs) for quantitation. Each porphyrin-NHS ester (1.0 mM) in aqueous solution (pH 9) containing two equivalents of amine at room temperature afforded >85% amidation product in <2 hours. The results provide a quantitative foundation for effective bioconjugation of water-soluble porphyrins in dilute aqueous solution.

Results

The two porphyrins chosen are shown in Chart 1. The numbering system from the synthesis paper[1] is retained here. Each porphyrin contains a 2,6-bis[$HO_2C(CH_2CH_2O)_7$]phenyl group for water-solubilization. The short PEG groups project above and below the face of the porphyrin macrocycle (as shown by ^{1}H NMR measurements[1]), and the carboxylic acid termini are expected to be predominantly ionized at neutral or basic pH values. Each porphyrin also bears an NHS ester. In porphyrin **P3-NHS**, the ester is derived from an aryl carboxylic acid, whereas in **P4-NHS**, the ester is derived from an alkanoic acid. The two porphyrins were examined to assess stability toward hydrolysis (in liquid solution), stability on storage (in frozen solution), and reactivity toward an amine to afford the corresponding amide. The latter reactions were examined as a function of pH, concentration, and temperature. All of the studies were carried out multiple times over a period of months. The following results are from single, representative analyses.

Stability of porphyrin NHS-esters toward hydrolysis

How rapidly do porphyrin-NHS esters undergo hydrolysis during conditions of bioconjugation? To address this question, porphyrins **P3-NHS** and **P4-NHS** (1.0 mM each) were subjected to a series of hydrolysis tests at room temperature in an aqueous solution at pH values of 8.0, 8.5, or 9.0 (Fig. 1, panel A). The samples were prepared by the addition of 1 part of a dimethylsulfoxide (DMSO) solution (containing 0.05% formic acid) of the porphyrin-NHS ester to 9 parts of a 50 mM $NaHCO_3/Na_2CO_3$ buffer, affording an aqueous solution that contained 10% DMSO, 0.005% (1.1 mM) formic acid, and 45 mM carbonate buffer. The formic acid was included to neutralize any adventitious base and thereby stabilize the solution; among all acids, formic acid was chosen given its use in the mass spectrometric analyses. All samples for hydrolysis or amidation studies reported herein were prepared in the same manner. The reaction mixture hereafter is referred to as "carbonate buffer/10% DMSO".

The reactions at pH 8.0, 8.5, or 9.0 were monitored by HPLC (5% to 95% acetonitrile in water with 0.1% formic acid over 9 minutes) with online MS-Abs analysis. Examination of the hydrolysis reaction mixture of **P3-NHS** showed three significant peaks (t_R = 5.2, 5.5, and 5.8 min) at 405 nm, the peak of the porphyrin Soret band (Fig. 1, panel B). The starting porphyrin **P3-NHS**, the slowest eluting component, gave m/z = 655.2 (z = 2). Mass analysis led to assignments of the other peaks as **P3-X** (5.2 min, m/z = 664.1 (z = 2); net +18 Da) and the hydrolyzed porphyrin-carboxylic acid **P3** (5.5 min, m/z = 606.6 (z = 2); net −97 Da). One possible structure of **P3-X** entails ring-opening of the succinimidyl unit as shown in the inset to Fig. 1, panel B. A similar ring-opening byproduct (*vide infra*) was reported by Eisenhut *et al.*[3] No further separation nor investigation has been conducted for **P3-X**. In the case of **P4-NHS**, similar components were identified by HPLC-MS-Abs (Fig. 1, panel C) at 5.3 min (**P4-X**), 5.6 min (**P4**), and 5.9 min (**P4-NHS**).

The rate of hydrolysis of **P3-NHS** (1.0 mM) was examined at the three pH values. The results are plotted in Fig. 2, panel A. The half-life ($t_{1/2}$) for the NHS ester was found to be 210, 180, or 125 min at pH 8.0, 8.5, or 9.0, respectively. The risetime for formation of the hydrolyzed product **P3** was slower than the rate of consumption of **P3-NHS** (Fig. 2, panel B), while the amount of **P3-X** rose and then declined over 12 to 48 h. Taken together, the results suggest that unknown **P3-X** could be an intermediate on the path to **P3**. Similar trends were observed for **P4-NHS** (1.0 mM): the hydrolysis $t_{1/2}$ was 190, 130, or 110 min at pH 8.0, 8.5, or 9.0, respectively (Fig. 2, panel C). Compared with **P3-NHS**, the unknown peak assigned to **P4-X** remained as a minor component at all pH values examined (Fig. 2, panel D).

Additional studies showed that the rate of formation of **P3-X** increased as the pH was increased, and exceeded that of **P3** at pH 9.0 (Fig. 3, panels A–C). Similar results were observed for the rate of formation of **P4-X** (Fig. 3, panels D–F).

A further experiment addressed the stability of the porphyrin-NHS esters on standing in frozen solution at −20 °C. Stock solutions (10 mM each) were prepared in DMSO containing 0.20%, 0.10%, or 0.05% formic acid and immediately cooled to −20 °C, considerably below the freezing point of DMSO (19 °C). The stock solution of **P4-NHS** containing 0.20% or 0.10% formic acid exhibited ~1% hydrolysis over 24 h, while that containing

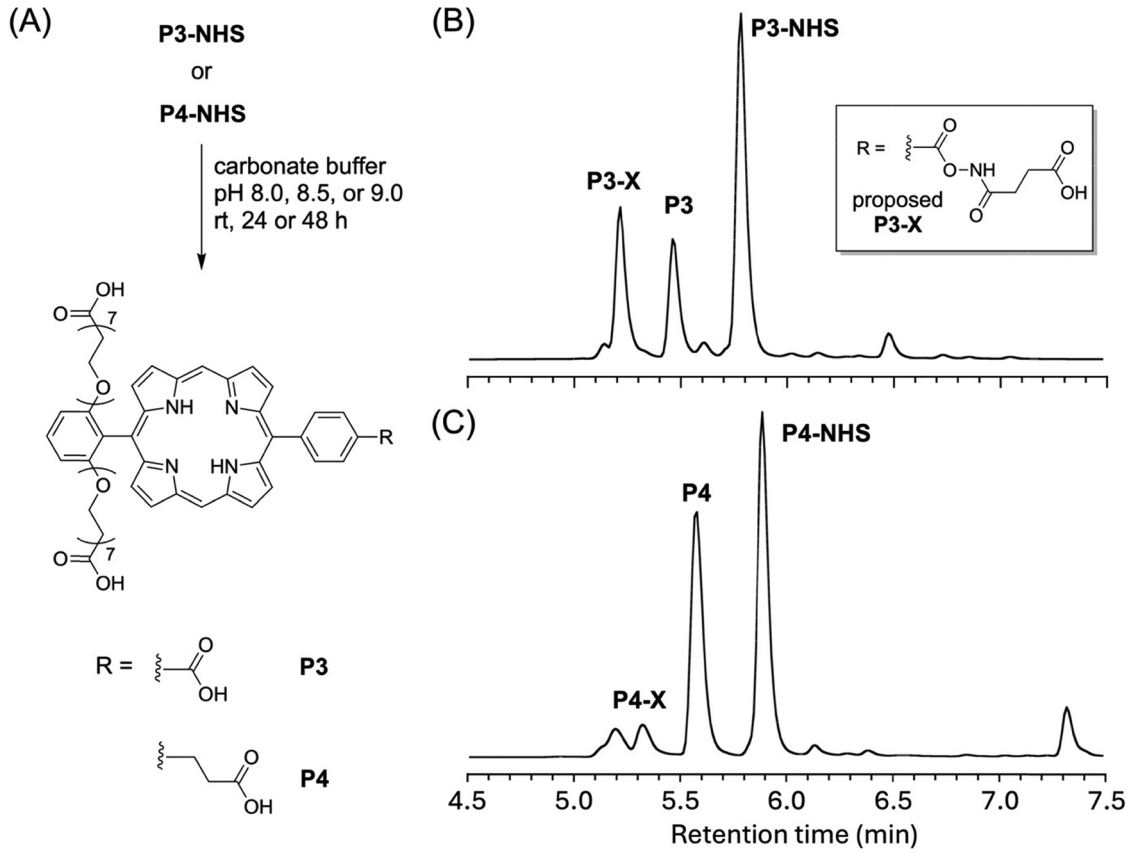

Fig. 1 (A) Hydrolysis of **P3-NHS** and **P4-NHS** in carbonate buffer/10% DMSO. Panels B and C: Reversed-phase C18 HPLC trace with absorption spectral detection of crude hydrolysis mixtures (50 mM, pH 9.0) after 2 h at room temperature for **P3-NHS** (B) or **P4-NHS** (C).

0.05% formic acid showed 3.9% hydrolysis over 24 h. Over 2 weeks, during which the samples were repeatedly (days 0, 1, 3, 5, 7, and 14) thawed and refrozen (within 15 min), **P4-NHS** showed 11%, 14%, or 15% hydrolysis for 0.20%, 0.10%, or 0.05% formic acid, respectively. In contrast, **P3-NHS** was more stable: $<1\%$ hydrolysis after 24 h, and $<2\%$ hydrolysis over 2 weeks, regardless of the formic acid concentration. While any hydrolysis is unwanted, the low level indicates both porphyrin-NHS esters can be stored for at least several days. These results provide valuable information for designing more stable porphyrin-based compounds for bioconjugation.

Amidation of porphyrin NHS-esters

Conjugation reactions were carried out of porphyrin-NHS esters (**P3-NHS**, **P4-NHS**) with a short amino-PEG reagent, H_2N–$(CH_2CH_2O)_4$–Me (denoted $mPEG_4$-NH_2), as a surrogate for a biomolecule (Fig. 4, panel A). The conjugations were carried out with each porphyrin-NHS ester at 1.0 mM and 2 equivalents of $mPEG_4$-NH_2 (2.0 mM) and examined at the three pH values at room temperature in carbonate buffer/10% DMSO. In each case, the porphyrin-amide-PEG **P3-PEG** or **P4-PEG** was observed as a major component within 15 min. Illustrative examples of the reaction after 15 minutes at pH 9.0 are provided in Fig. 4, panel B and panel C. For **P3-NHS**, the reaction was approximately half-complete on the basis of the peak intensities for **P3-PEG** (5.35 min) and **P3-NHS** (5.8 min). Note that the peak of **P3** upon HPLC analysis appeared at $\sim$5.45 min, and over the course of 21 HPLC runs (different reactions and various time-points), was generally found to be either half-overlapped or completely hidden by the peak of **P3-PEG**. A small amount of the unknown species **P3-X** (5.15 min. 5–7% yield) also was detected in all traces. Because the prior hydrolysis study showed a similar rate of formation of **P3** and the byproduct **P3-X** in the first 4–6 hours, the final yield of **P3** in this conjugation study was estimated to be 5–10%. This estimation was substantiated by a separate study that employed a modified HPLC condition (*vide infra*). For **P4-NHS**, the reaction was faster, and the ratio of product **P4-PEG** (5.4 min) to starting material **P4-NHS** (5.9 min) was about 2.5 : 1. Here, a small amount of hydrolyzed product **P4** (5.55 min) was detected, albeit partially overlapped with the peak from **P4-PEG**.

For confirmation of the HPLC analyses, the two porphyrins (**PS-NHS**, **P4-NHS**) were reacted at small scale (6.0 mg each) with $mPEG_4$-NH_2 (at room temperature in carbonate buffer/10% DMSO at pH 9.0) to give the corresponding porphyrin-amide-PEG (**P3-PEG**, **P4-PEG**) in 87% or 91% yield, respectively. Each porphyrin was characterized by ^{1}H NMR spectroscopy, absorption spectroscopy, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), and electrospray ionization mass spectrometry (ESI-MS). The ^{1}H NMR spectral data

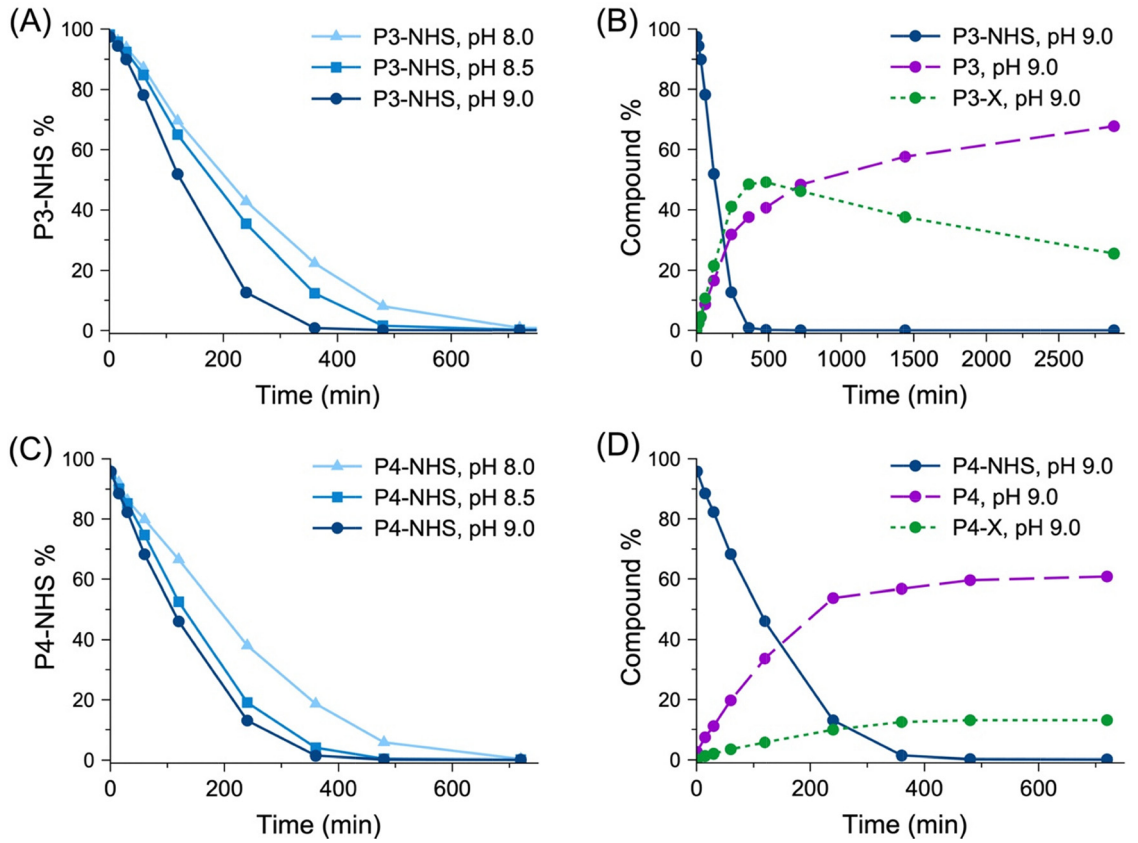

Fig. 2 Kinetics of hydrolysis of porphyrin-NHS esters in carbonate buffer/10% DMSO at given pH values at room temperature, assessed by HPLC-MS-Abs. (A) **P3-NHS** at three pH values over 12 h; (B) three major components of **P3-NHS** hydrolysis at pH 9.0 over 48 h; (C) **P4-NHS** at three pH values over 12 h; (D) three major components of **P4-NHS** hydrolysis at pH 9.0 over 12 h.

of **P3-PEG** and **P4-PEG** showed the typical chemical shifts (as observed for analogues in the companion paper[1]) for the aryloxy-PEG groups projected above and below the face of the porphyrin. The chemical shifts arise from orientation of the groups in the ring current of the porphyrin macrocycle. The NMR data are provided in the ESI.†

The absorption spectra of porphyrins **P3-NHS**, **P3**, and **P3-PEG** are shown in Fig. 5 panel A. The absorption spectra of porphyrins **P4-NHS**, **P4**, and **P4-PEG** are shown in Fig. 5 panel B. All six spectra were collected in DMSO at room temperature (for tabulated spectral data, see the companion paper[1]). In each set, the Soret bands are essentially identical (as expected), indicating the absence of spectral changes due to the presence of various substituents. Accordingly, absorption spectroscopy is a viable method for quantitative evaluation of the relative yields of porphyrins.

The rates of the conjugation of the amine reagent $mPEG_4$-NH_2 (2.0 mM) with the porphyrin-NHS esters (**P3-NHS**, **P4-NHS**, 1.0 mM each) were examined at the three pH values. The results are shown in Fig. 6. For **P3-NHS**, the half-time for formation of the amide conjugate ($t_{1/2}$) was found to be 80, 20, or 10 min at pH 8.0, 8.5, or 9.0, respectively (Fig. 6, panel A). The conjugation rate was more sensitive to pH *versus* that of the hydrolysis reaction. The rate of formation of **P3-PEG** mirrored the rate of consumption of **P3-NHS**, with **P3-X** observed as a minor species. Because the peaks of **P3-NHS** and **P3** were overlapped, the entire **P3-NHS** peak was integrated for yield calculation and graph plotting in Fig. 6 and Fig. 7. The total yield of **P3-PEG** and **P3** was approximately 91–93%, and the yield of **P3-PEG** was estimated to be 80–85%. For **P4-NHS**, the $t_{1/2}$ was 25, 10, or 5 min at pH 8.0, 8.5, or 9.0, respectively (Fig. 6, panel B). The final yield of **P4-PEG** was approximately 87–92%. Although the hydrolysis rate of **P4-NHS** was faster at pH 9.0 (*versus* pH 8.5 or 8.0), the conjugation reaction at pH 9.0 provided the highest yield of **P4-PEG** and lowest relative yield of **P4**. The rate differences for the conjugations with **P3-NHS** and **P4-NHS** as a function of pH are clearly seen in direct overlays (Fig. 7).

To further investigate the effects of conditions on the amidation process, the reaction of the amine $mPEG_4$-NH_2 (2.0 equivalents) with the porphyrin-NHS esters (**P3-NHS**, **P4-NHS**) was examined in carbonate buffer (50 mM, pH 9) at different temperatures and concentrations (Table 1). A modified HPLC condition – elution with acetonitrile (30% to 55% over 7 min, then 55% to 95% over 2 min) in water containing 0.1% formic acid – enabled resolution of the major peaks (Fig. 8, panel A). In this manner, the ratios could be calculated on the basis of peak integration, which also increased the accuracy of the yield of the porphyrin-amide-PEG product *versus* the prior set of studies (Fig. 6 and Fig. 7). The results are provided in Table 1.

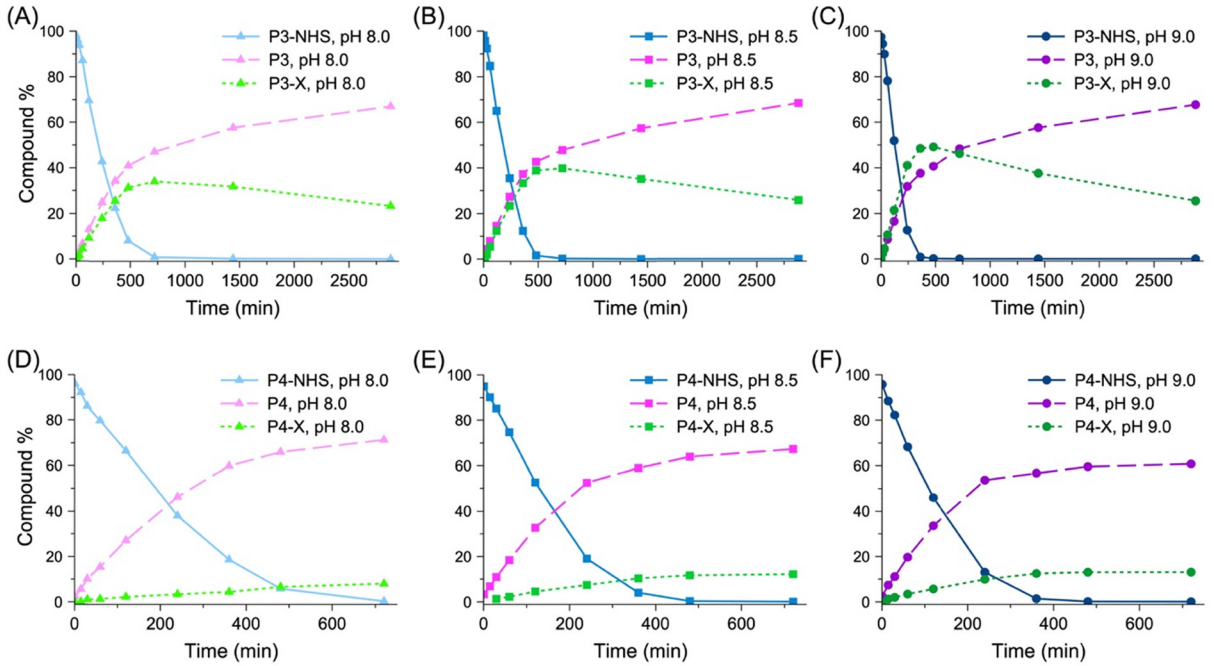

Fig. 3 Analysis of the hydrolysis of porphyrin NHS-esters **P3-NHS** in carbonate buffer/10% DMSO at various pH values at room temperature, assessed by HPLC-MS-Abs. Panels A–C, data for **P3-NHS** over 48 h. Panels D–F, data for **P3-NHS** over 12 h.

The results show that both **P3-NHS** and **P4-NHS** gave a higher conjugation yield at room temperature (*versus* 37 °C) and at higher concentration. Porphyrin **P4-NHS** gave a higher conjugation yield than **P3-NHS** under the same conditions (Fig. 8, panel B). The unknown species **P3-X** was still observed as a major byproduct in the crude mixture in all amidation reactions, forming in larger relative quantities at lower temperature and lower concentrations. On the other hand, the yield of hydrolysis product porphyrin-carboxylic acid **P3** was higher at lower reaction concentrations (same as **P3-X**) or when the temperature increased (opposite to **P3-X**). The same trend was observed for **P4-X**, which was a minor species in all amidation reactions of **P4-NHS**. The results augur well for application of **P3-NHS** and **P4-NHS** as bioconjugatable porphyrins for life sciences studies.

Discussion

The results with the present porphyrin-NHS esters (**P3-NHS**, **P4-NHS**) can be placed in context. The conjugation with NHS esters occupies a large swath of bioconjugation chemistry.[4] Most papers report application of the bioconjugation rather than in-depth studies of the effects of conditions on kinetics and yields. A search for quantitative studies of bioconjugation was performed – albeit without a sense of completeness – given the vast scope of the bioconjugation literature, which spans >50 years and encompasses a remarkable diversity of biomolecules and reactants. A number of studies for discussion here (Table 2) were chosen because quantitative analysis was performed of reactions with well-defined conditions including specified reactant ratios and concentrations. In general, in each case, either the amine or the NHS ester was used in substantial excess, as seen by inspection of columns 3 and 5 in the table. By contrast, all studies herein used only 2 equivalents of the amine reactant $mPEG_4$-NH_2 and typically 1.0 mM of the porphyrin-NHS ester.

Further perspective is provided by the reports cited in entries 1–7 in Table 2:

(1) Cuatrecasas and Parikh reported that the optimal pH for amidation of amino acids differs with the respective amine pK_a values.[5]

(2) Lomant and Fairbanks reported that the $t_{1/2}$ for hydrolysis of a bis(alkyl-NHS-ester) was 4–5 h at 0 °C in phosphate buffer (5 mM, pH 7.0).[6]

(3) Cline and Hanna determined rate constants for amidation and hydrolysis with NHS esters. The hydrolysis was first-order in [OH^-] under pH 7.6–11.1 and independent of buffer concentration, while the amidation was first-order in $[amine]_{free}$ (based on the pK_a of the amine and pH of the solution) with rate constant independent of pH.[7]

(4) Hosoda *et al.* reported that the $t_{1/2}$ for hydrolysis of a steroidal-NHS ester at pH 8.0 was 36 min.[8]

(5) Eisenhut *et al.* employed a bioconjugatable linker (**BAT-NHS**) and isolated a byproduct with m + 18, for which a ring-opened ester (**BAT-X**) was proposed (Chart 2).[3] The *N–O* alkyl ester (succinimidyloxy) motif of **BAT-X** is identical to that proposed herein for **P3-X** and **P4-X**. Among the entries in Table 2, there are only two cases (entries 3 and 5) where an aryl-NHS ester was employed.

(6) Nojima *et al.* reported that the hydrolysis rate of a 20-kDa or a 40-kDa PEG-NHS ester at pH 9.0 was 14- or 33-fold higher

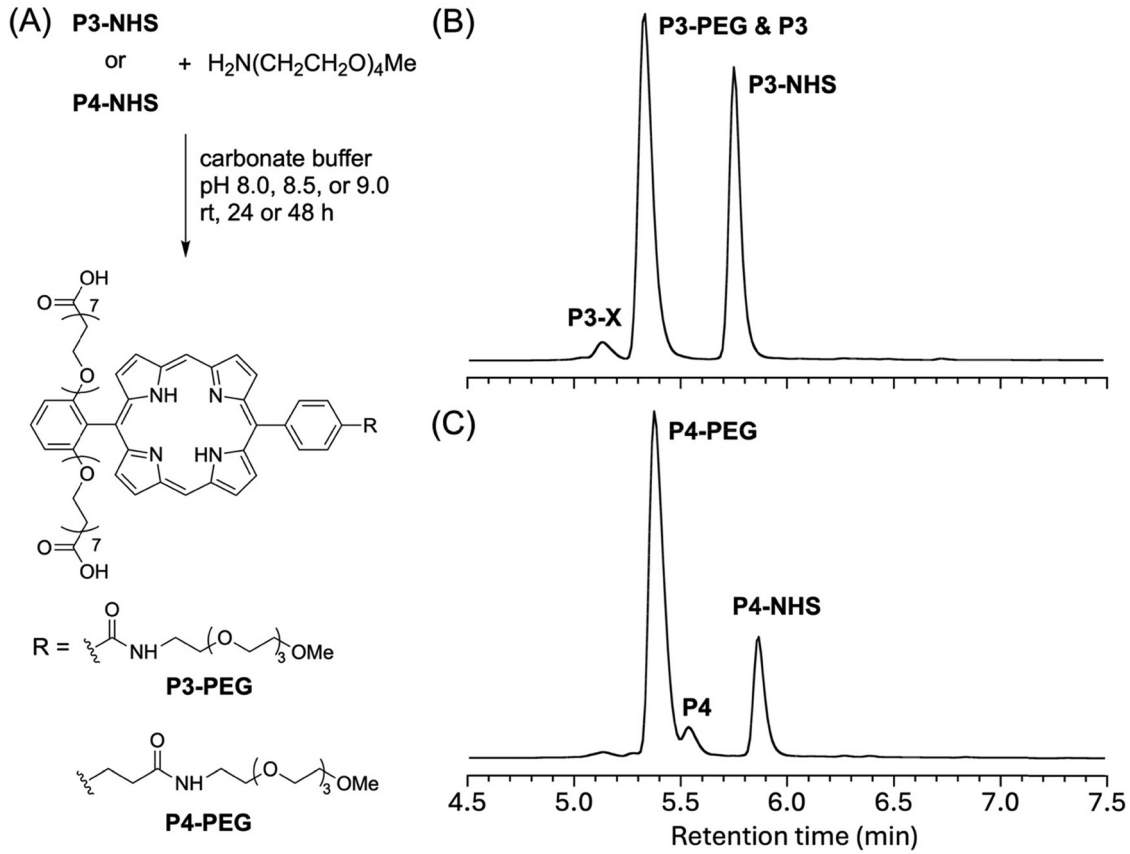

Fig. 4 (A) Conjugation of **P3-NHS** or **P4-NHS** with $mPEG_4$-NH_2 in carbonate buffer/10% DMSO at room temperature. Panels B and C: Reversed-phase C18 HPLC trace with absorption spectral detection of the crude conjugation mixture with $mPEG_4$-NH_2 after 15 min (pH 9.0) at room temperature for **P3-NHS** (B) or **P4-NHS** (C).

than at pH 7.4, respectively. The amidation reaction rate of both PEG-NHS reagents with bovine lactoferrin was dependent on pH, temperature, concentration and molar ratios.[9]

(7) Bjerneld *et al.* reported four pathways for consumption by proteins of NHS reagents: hydrolysis, amidation with Tris buffer, amidation at the protein *N*-terminus, and amidation of the lysine ε-amine.[10] On the other hand, Cuatrecasas *et al.* discovered that the cysteine sulfhydryl competes with amines for NHS conjugation while the imidazole group of histidine accelerates the hydrolysis.[5] Mädler *et al.* also proposed that arginine could facilitate the reaction of the hydroxyl group of serine, threonine, or tyrosine with the NHS crosslinking reagent.[11]

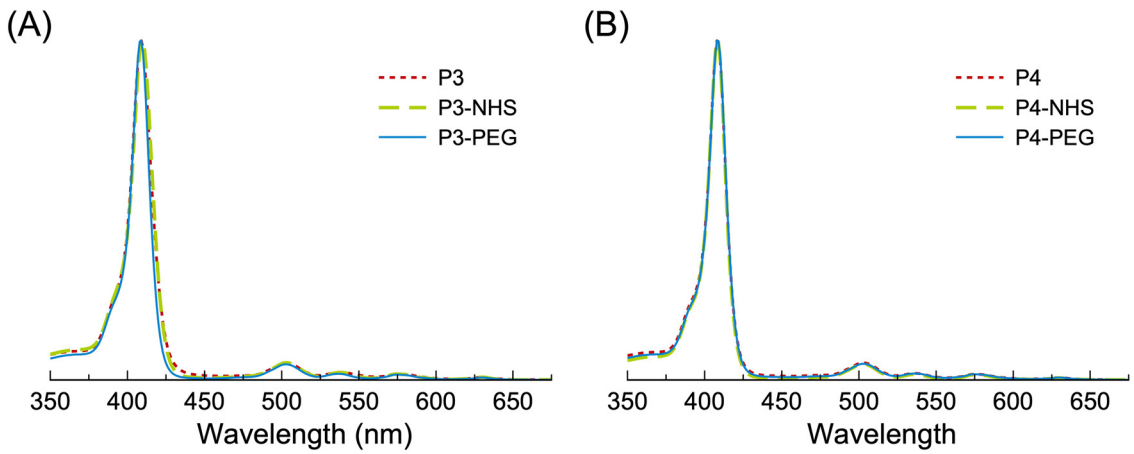

Fig. 5 Absorption spectra of porphyrins (normalized at the respective Soret bands) in DMSO at room temperature: (A) **P3** (dotted red trace), **P3-NHS** (dashed green trace), and **P3-PEG** (solid blue trace). (B) **P4** (dotted red trace), **P4-NHS** (dashed green trace), and **P4-PEG** (solid blue trace).

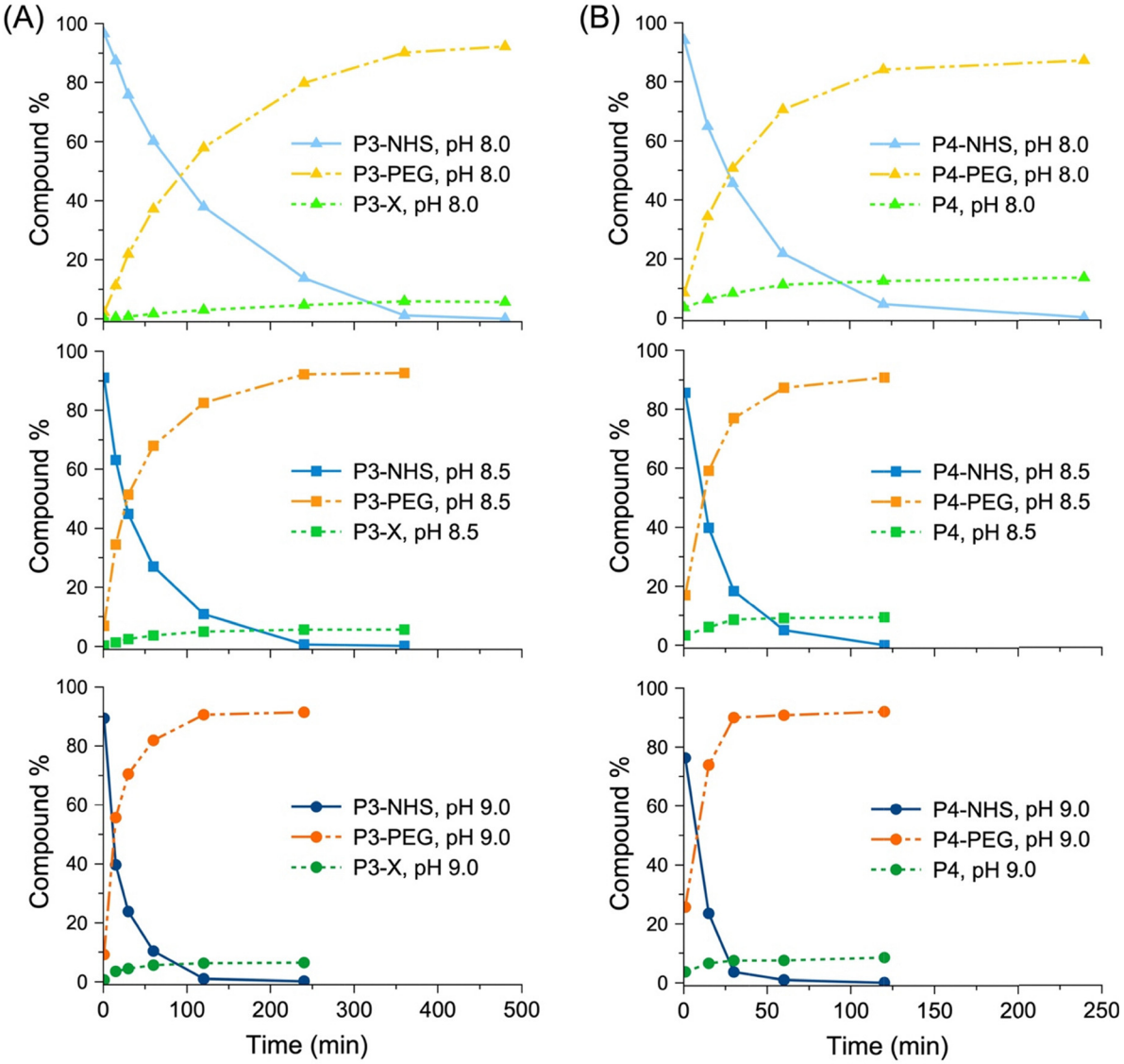

Fig. 6 Kinetics of conjugation of porphyrin-NHS esters with $mPEG_4$-NH_2 in carbonate buffer/10% DMSO at various pH values at room temperature, assessed by HPLC-MS-Abs. (A) Data for **P3-NHS**; (B) data for **P4-NHS**.

Finally, across diverse bioconjugation reactions, mixed aqueous organic solutions are commonplace, including use of *p*-dioxane,[7,8] DMSO,[6,10] DMF,[3,6] acetone,[6] *etc.* The rate of amidation in general is faster than that of hydrolysis; however, in selected circumstances, such as upon reaction with amines on surfaces, the hydrolysis may prevail.[12] The medium for the studies reported here – comprised by adding 1 part of porphyrin-NHS ester in DMSO (containing 0.05% formic acid) to 9 parts of carbonate buffer (50 mM) – fit well within that regime.

Conclusions

The literature for bioconjugation often concerns reactions of an NHS-ester with a protein. Interpreting the origins of limited bioconjugation efficacy can be difficult because (1) the number and thus concentration of the amines available for reaction in a globular protein often are not clear, and (2) the competitive hydrolysis rate of the NHS-ester is not known for the conditions under study. The studies of the porphyrin NHS-esters examined here focused on defined conditions with only a 2-fold ratio of the amine reagent to the porphyrin-NHS ester in dilute aqueous solution at mild temperature.

The work reported here and in the preceding (companion) paper together have evaluated porphyrins *via* three filters: (1) synthetic accessibility, (2) water solubility, and (3) bioconjugation efficacy. A key objective was to access molecular designs that could be used without reliance on click chemistry. The benzoate and phenylpropanoate NHS esters were found to be superior upon surveying several types of linkers and potential conjugatable entities. For both porphyrin-NHS esters under the conditions examined, the rate of hydrolysis was quite fast, but in each case, the rate of amidation was much faster. The conditions employed a porphyrin-NHS ester at 1.0 mM and afforded the amide in ~90% (or greater) yield. Decreasing the

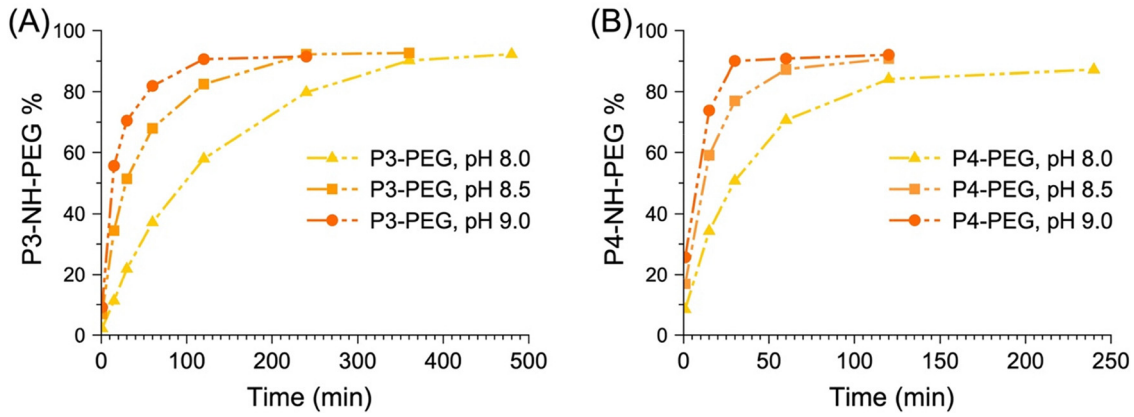

Fig. 7 Kinetics of conjugation reactions of porphyrin-NHS esters with $mPEG_4$-NH_2 in carbonate buffer/10% DMSO at various pH values at room temperature, assessed by HPLC-MS-Abs. (A) Conjugations of **P3-NHS**; (B) conjugations of **P4-NHS**.

porphyrin concentration to 0.1 mM still resulted in at least 56% yield. The use of a porphyrin-NHS ester at 0.1–1.0 mM in carbonate buffer/10% DMSO at pH 9.0 at room temperature should accommodate the solubility constraints of porphyrins while affording rapid and high yielding amidation, and hence be broadly applicable for diverse bioconjugation processes.

Experimental section

General methods

Commercially available compounds were used as received. Silica (SiliaFlash® P60, 40–63 μm, Silicycle, R12030B) was used for column chromatography. ^{1}H NMR (700 MHz) spectra were recorded in CD_2Cl_2 at room temperature. Chemical shifts for ^{1}H NMR spectra are reported in parts per million (δ) relative to tetramethylsilane or a solvent signal [CD_2Cl_2, δ = 5.32 ppm].

Table 1 Conjugation of porphyrin-NHS esters with $mPEG_4$-NH_2 at pH 9 under different temperature and concentrations[a]

Substrates	Entry	T^b (°C)	$[C]^c$ (μM)	Por-X[d]	Por-PEG[d]	Por-COOH[d]
P3-NHS	1	19	1000	2.6	88.4	9.4
	2	19	316	10.2	74.0	15.1
	3	19	100	20.3	55.9	21.6
	4	37	1000	0.4	86.6	13.3
	5	37	316	2.2	71.8	25.8
	6	37	100	5.5	47.9	45.1
P4-NHS	7	19	1000	1.0	97.0	13.7
	8	19	316	2.7	89.4	18.2
	9	19	100	6.5	73.2	27.5
	10	37	1000	0.8	95.8	15.1
	11	37	316	1.1	86.8	22.4
	12	37	100	1.7	66.8	39.3

[a] The reaction was carried out in carbonate buffer/10% DMSO. [b] Room temperature was ~19 °C. [c] The listed numbers are the final concentration of porphyrin-NHS ester in the reaction. The PEG reagent concentration was twice that of the porphyrin in each case. [d] The percentage of each species (Por-X stands for **P3-X** or **P4-X**; Por-PEG stands for **P3-PEG** or **P4-PEG**; Por-COOH stands for **P3** or **P4**) was identified and estimated by HPLC-MS-Abs at 404 nm. The eluent was acetonitrile (30% to 55% over 7 min, then 55% to 95% over 2 min) in water containing 0.1% formic acid.

MALDI-MS was performed using the matrix α-cyano-4-hydroxycinnamic acid (α-CHCA). Absorption spectra were collected in MeOH or DMSO at room temperature. ESI-MS data are reported for the molecular ion or cationized molecular ion. High-performance liquid chromatography–mass spectrometry (HPLC-MS) was performed on a Shimadzu LCMS-2020 equipped with a Shim Pack XR-ORS (reversed-phase C18, 2.2 μm, 3.0 × 50 mm) column and SPD-M40 photo diode array (PDA) detector. The data were recorded and processed with Labsolutions software (Shimadzu, ver. 5.1113) under Windows 10 (Microsoft).

Molar absorption coefficient value

Each porphyrin here is assumed to have a molar absorption coefficient for the Soret band (at ~405 nm) of 400 000 M^{-1} cm^{-1}. The value is slightly less than that of *meso*-tetraphenylporphyrin, for which reported values range from <100 000 to >1 000 000 M^{-1} cm^{-1}.[13] The use of a common value across all porphyrins herein provides a consistent framework for the studies. All of the studies derive from stock solutions prepared in the same manner with use of the same molar absorption coefficient. The absorption spectra of the porphyrins – starting materials **P3-NHS** and **P4-NHS**, intermediates, and products – are nearly identical with each other, supporting the use of a common value. We note that should a lower value of the molar absorption coefficient be identified, the ratio of porphyrin/PEG-amine employed herein would be larger. For example, a molar absorption coefficient of 200 000 M^{-1} cm^{-1} would correspond to a 1 : 1 ratio of porphyrin/PEG-amine actually employed rather than 1 : 2 as has been inferred with the assumed value of 400 000 M^{-1} cm^{-1}. Similarly, should the molar absorption coefficient be found to be greater than 400 000 M^{-1} cm^{-1}, the ratio actually employed would be <1 : 2.

Hydrolysis and conjugation study of porphyrin-NHS esters

A stock solution of **P3-NHS** (10 mM) or **P4-NHS** (10 mM) was prepared in a solution of DMSO containing 0.05% formic acid. The porphyrin concentration was calculated on the basis of the molar absorption coefficient (400 000 M^{-1} cm^{-1}) at 405 nm (**P3-NHS**) or 404 nm (**P4-NHS**). A stock solution of H_2N–$(CH_2CH_2O)_4$–Me (100 mM) was prepared in deionized water. The $NaHCO_3$/

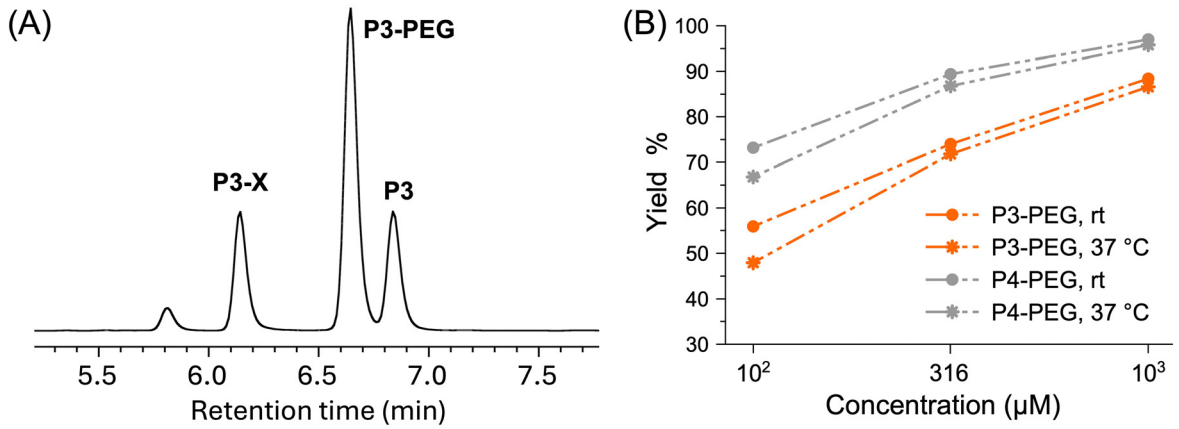

Fig. 8 (A) Reversed-phase C18 HPLC trace with absorption spectral detection of the crude conjugation mixtures of **P3-NHS** and $mPEG_4$-NH_2 (entry 3, 100 μM, pH 9.0) in carbonate buffer/10% DMSO after 2 days. (B) Yield comparison among different porphyrin-NHS esters at different temperature and concentrations (log scale).

Table 2 Representative studies of amidation of NHS esters

Entry	R–NH_2	[R–NH_2][a]	R′–NHS	[R′–NHS]	Conditions[b] pH (buffer)	Method[c]
1[5]	Amino acids	0.5–100 mM	Agarose alkyl	~1/2–1/400 eq.	6.3 (NaOAc), 4 °C 8.6 ($NaHCO_3$), 4 °C	Amino acid analyzer
2[6]	Hemoglobin	3.1 μM	Bis(alkyl-NHS)	80, 200, 500 μM	7.0 (PBS), 0 °C	SDS-PAGE
3[7]	Diverse amines	3–500 mM	Anisoyl-NHS	50 μM	6.0–11.0 (dioxane/water)	HPLC-Abs 254 nm
4[8]	Glycine	667 mM	Alkyl-NHS	167 mM	6.2 (PBS), 4 °C 7.3 (PBS), 4 °C 8.0 (PBS), 4 °C	TLC scanner
5[3]	mAb	19 or 38 μM	Aryl	228 μM	8.0 (PBS) 8.5 (PBS)	HPLC-Abs 270 nm
6[9]	Lactoferrin	6.5 μM	PEG alkyl	65 μM	6.0–8.0 (PBS) 9.0 (borate)	SDS-PAGE
7[10]	Diverse proteins	5–71 μM	Cy5 alkyl	7–130 μM	8.7 (Tris)	SDS-PAGE

[a] Reagent concentration refers to the molarity of the molecular species, such as a small molecule or protein. The concentration of reactive sites (*e.g.*, lysine-NH_2 residues) may be much greater and depends on each protein. [b] Reactions were conducted at room temperature (20–25 °C) unless otherwise specified. [c] Separation (detection) method to evaluate the components ratio and reaction rate.

Na_2CO_3 buffer (50 mM) was adjusted to pH 8.0, 8.5 or 9.0. Analysis was carried out of the following reaction processes by injection of samples (0.2 μL) into an HPLC equipped with MS-Abs detection modalities.

For the hydrolysis study. A sample of 5.0 μL of each porphyrin-NHS ester was mixed with 45 μL of the carbonate buffer in an HPLC vial. A small portion (0.2 μL) of the reaction mixture was analyzed by HPLC at certain time points (1 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h). In each HPLC run, detection was carried out absorption spectroscopy as described above. The peaks of the various species were identified and integrated. By comparison of the integrated areas, the relative yield of the set of species within a given run was calculated.

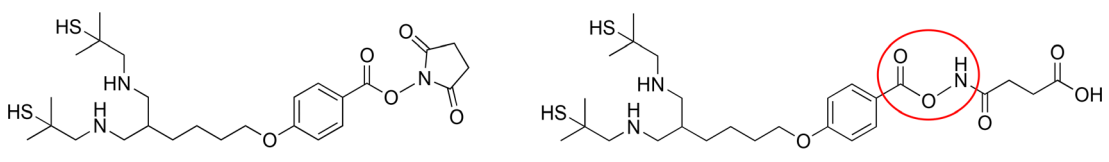

Chart 2 Ring-opening hydrolysis product (**BAT-X**) proposed by Eisenhut *et al.*[3]

For the conjugation study at different pH. A sample of 1.0 μL of the H_2N–$(CH_2CH_2O)_4$–Me stock solution was pre-mixed with 44 μL of the carbonate buffer in an HPLC vial, then treated with 5.0 μL of the porphyrin-NHS ester stock solution. A small portion (0.2 μL) of the reaction mixture was analyzed by HPLC at certain time points (1 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h). In each HPLC run, detection was carried out by absorption spectroscopy as described above. The peaks of the various species were identified and integrated. By comparison of the integrated areas, the relative yield of the set of species within a given run was calculated.

For the conjugation study at different temperature and concentrations. The stock solution of a porphyrin-NHS ester was diluted (in DMSO containing 0.05% formic acid) to 3.16 mM or 1.0 mM, and the H_2N–$(CH_2CH_2O)_4$–Me stock solution was diluted (in deionized water) to 31.6 mM or 10 mM. A sample of 1.0 μL of each H_2N–$(CH_2CH_2O)_4$–Me solution (the stock solution and the two diluted solutions) was pre-mixed with 44 μL of the carbonate buffer (pH 9.0) in an HPLC vial, then treated with 5.0 μL of the corresponding porphyrin-NHS ester solution (stock solution and two diluted solutions). A small portion (0.2 μL) of each reaction mixture was analyzed by HPLC after 18 h (for reactions at 37 °C) or 48 h (for reaction at room temperature). In each HPLC run, detection was carried out absorption spectroscopy as described above. The peaks of the various species were identified and integrated. By comparison of the integrated areas, the relative yield of the set of species within a given run was calculated.

Synthesis

5-(2,6-Bis((1-hydroxy-1-oxo-4,7,10,13,16,19-hexaoxahenicosan-21-yl)oxy)phenyl)-15-(4-(2,5,8,11-tetraoxa-14-aza-15-oxopentadecan-15-yl)phenyl)porphyrin (P3-PEG). A solution of 5-(2,6-bis((1-hydroxy-1-oxo-4,7,10,13,16,19-hexaoxahenicosan-21-yl)oxy)phenyl)-15-(4-(*N*-succinimidyloxycarbonyl)phenyl)porphyrin (**P3-NHS**, 6.0 mg, 4.6 μmol) in DMSO + 0.05% formic acid (0.46 mL) was stirred at room temperature. After 2 min, a sample of H_2N–$(CH_2CH_2O)_4$–Me (1.9 mg, 9.2 μmol) in $NaHCO_3/Na_2CO_3$ buffer (50 mM) at pH = 9 (4.14 mL) was added. The reaction mixture was stirred for 5 h at room temperature, and then the solvent was removed under reduced pressure. The crude product was loaded on a silica pad (1 cm × 2 cm) and washed with CH_2Cl_2/MeOH (5 : 1, 40 mL) to MeOH to give an eluant that upon concentration afforded a red solid (5.6 mg, 87%): ^{1}H NMR (700 MHz, CD_2Cl_2) δ 10.30 (s, 2H), 9.41–9.38 (m, 4H), 9.03–9.01 (m, 4H), 7.75 (t, J = 8.9 Hz, 1H), 7.47 (s, 1H), 7.08 (d, J = 8.9 Hz, 2H), 4.04–3.99 (m, 4H), 3.82 (d, J = 2.4 Hz, 4H), 3.75 (dd, J = 5.9, 3.1 Hz, 2H), 3.71 (dd, J = 5.8, 3.1 Hz, 2H), 3.67 (dd, J = 5.9, 3.0 Hz, 2H), 3.63 (dd, J = 5.7, 3.0 Hz, 2H), 3.59 (dd, J = 5.6, 3.7 Hz, 2H), 3.50–3.30 (m, 26H), 3.29 (s, 3H), 3.15 (t, J = 4.5 Hz, 4H), 2.94–2.91 (m, 8H), 2.56–2.52 (m, 4H), 2.25–2.20 (m, 8H), 2.08 (t, J = 4.6 Hz, 4H), 1.85 (t, J = 4.6 Hz, 4H), −3.17 (d, J = 30.2 Hz, 2H); λ_{abs} (MeOH) 404, 500, 533, 573, 629 nm; λ_{abs} (DMSO) 409, 503, 537, 574, 629 nm; MALDI-MS obsd 1400.1, calcd 1399.7 [M^+]; ESI-MS obsd 1399.6572, calcd 1399.6569 [M^+], M = $C_{72}H_{97}N_5O_{23}$.

5-(2,6-Bis((1-hydroxy-1-oxo-4,7,10,13,16,19-hexaoxahenicosan-21-yl)oxy)phenyl)-15-(4-(2,5,8,11-tetraoxa-14-aza-15-oxoheptadecan-17-yl)phenyl)porphyrin (P4-PEG). A solution of 5-(2,6-bis((1-hydroxy-1-oxo-4,7,10,13,16,19-hexaoxahenicosan-21-yl)oxy)phenyl)-15-(4-(3-(*N*-succinimidyloxy)-3-oxopropyl)phenyl)porphyrin (**P4-NHS**, 6.0 mg, 3.7 μmol) in DMSO + 0.05% formic acid (0.37 mL) was stirred at room temperature. After 2 min, a sample of H_2N–$(CH_2CH_2O)_4$–Me (1.5 mg, 7.4 μmol) in $NaHCO_3/Na_2CO_3$ buffer (50 mM) at pH = 9 (3.33 mL) was added. The reaction mixture was stirred for 1 h at room temperature and then the solvent was removed under reduced pressure. The crude product was loaded on a silica pad (1 cm × 2 cm) and washed with CH_2Cl_2/MeOH (5 : 1, 40 mL) to MeOH to give an eluant that upon concentration afforded a red solid (4.9 mg, 91%): ^{1}H NMR (700 MHz, CD_2Cl_2) δ 10.28 (s, 2H), 9.40 (d, J = 4.4 Hz, 2H), 9.38 (d, J = 4.5 Hz, 2H), 9.07 (d, J = 4.4 Hz, 2H), 9.00 (d, J = 4.4 Hz, 2H), 8.17 (d, J = 7.4 Hz, 2H), 7.72 (t, J = 8.7 Hz, 1H), 7.66 (d, J = 7.3 Hz, 2H), 7.07 (d, J = 8.7 Hz, 2H), 6.58–6.56 (m, 1H), 4.00 (t, J = 4.6 Hz, 4H), 3.66–3.56 (m, 14H), 3.54–3.47 (m, 10H), 3.42–3.40 (m, 8H), 3.36 (t, J = 4.6 Hz, 4H), 3.32–3.30 (m, 5H), 3.22 (t, J = 3.8 Hz, 4H), 3.00 (t, J = 4.6 Hz, 4H), 2.95 (t, J = 4.6 Hz, 4H), 2.79 (dd, J = 9.0, 6.8 Hz, 2H), 2.64 (t, J = 4.5 Hz, 4H), 2.32–2.27 (m, 8H), 2.14–2.13 (m, 4H), 1.94 (t, J = 4.6 Hz, 4H), −3.16 (d, J = 37.3 Hz, 2H); λ_{abs} (MeOH) 404, 500, 532, 574, 629 nm; λ_{abs} (DMSO) 408, 503, 536, 574, 630 nm; MALDI-MS obsd 1428.0, calcd 1427.7 [M^+]; ESI-MS obsd 1427.6881, calcd 1427.6887 [(M + H)$^+$], M = $C_{74}H_{101}N_5O_{23}$.

Conflicts of interest

The authors declare competing financial interests.

Data avaialability

The electronic ESI† includes ^{1}H NMR and MALDI-MS spectra for new compounds. All other data are contained in the paper.

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