Supporting Information

Experimental

MALDI-TOF Analysis of Conjugates

A Bruker Microflex LRF was used to acquire the MALDI-TOF-MS data, in linear positive mode (laser 60 Hz, Ion Source 1: 19.5 kV, Ion Source 2: 18.15 kV, Lens: 7.00 kV, Pulsed Ion Extraction 240 ns, Detector Gain 2850 V). Data were processed using Bruker flexAnalysis v3.4. A saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid, 20 mg) acetone (500 µL) was prepared as "Mix 1". A precursor saturated solution of CHCA (20 mg) in 70% acetonitrile with 5% formic acid (aq, 500 µL) was prepared alongside a saturated solution of DHB (2,5-dihydroxybenzoic acid, 20 mg) in 70% acetonitrile with 0.1% trifluoroacetic acid (aq, 500 µL). Solutions were prepared at room temperature and vortexed thoroughly for 60 seconds before use. 100 µL of DHB and CHCA solutions were then mixed (1:1) to provide "Mix 2". 0.5 µL of the CHCA/acetone "Mix 1" was spotted to a polished steel target plate (Bruker) and evaporated quickly to leave a thin layer of CHCA. 0.5 µL of protein sample (typically 10 µM in PBS [pH 7.2]) was spotted directly onto the layer. Then, 0.5 µL of "Mix 2" was added to the liquid droplet and the spot allowed to dry. Where reduced protein samples were used, an appropriate volume of DTT (dithiothreitol, 100 mM) was added to achieve a concentration of 10 mM and incubated for 30 min at 60 °C with gentle shaking (450 rpm).

NMR Spectroscopy

^{1}H NMR spectra were recorded with a Bruker Advance III HD 700 MHz spectrometer. Chemical shifts are given in ppm referenced to internal standards. The products were characterised as mixtures of 1,4- and 1,5-disubstituted triazole regioisomers, as is typical for SPAAC reactions.

The ^{1}H NMR spectra reflect the combined contributions of both regioisomers, with characteristic aliphatic and aromatic signals and integration consistent with the expected structures. In both cases, signals for NH and SO_3H were absent, likely due to exchange in D_2O.

*SPAAC product **4** (following SPAAC reaction between sulfo-DBCO amine (**1**) and 1-azido-1-deoxy-β-D-glucopyranoside):* ^{1}H NMR δ_H(700 MHz, D_2O): 7.78–7.20 (m, 9H, 8 × DBCO aromatic H, 1 × NH_2, reduced intensity, expected to exchange with D_2O), 5.99–5.78 (m, 1H), 5.43–5.32 (m, 1H), 4.65–4.45 (m, 2H), 4.07–4.02 (m, 1H, OH), 3.95–3.91 (m, 1H, OH), 3.90–3.74 (m, 2H, 2 × OH), 3.73–3.63 (m, 2H), 3.56–3.51 (m, 2H, including 1H from CH_2), 3.46–3.41 (m, 1H), 3.31–3.17 (m, 7H, 3 × CH_2, including 1H from CH_2), 1.23–1.11 (m, 1H, NH_2, reduced intensity, expected to exchange with D_2O).

*SPAAC product **5** (following SPAAC reaction between sulfo-DBCO amine (**1**) and 3-azido-L-alanine):* ^{1}H NMR δ_H(700 MHz, D_2O): 7.71–7.25 (m, 9H, 8 × DBCO aromatic H, 1 × NH_2, reduced intensity, expected to exchange with D_2O), 5.91–5.55 (m, 1H), 5.16–4.92 (m, 2H), 4.66–4.31 (m, 2H), 4.22–4.06 (m, 1H), 4.06–3.97 (m, 2H), 3.65–3.51 (m, 2H), 3.41–3.24 (m, 2H), 1.25–1.12 (m, 1H, NH_2, reduced intensity, expected to exchange with D_2O).

Figures

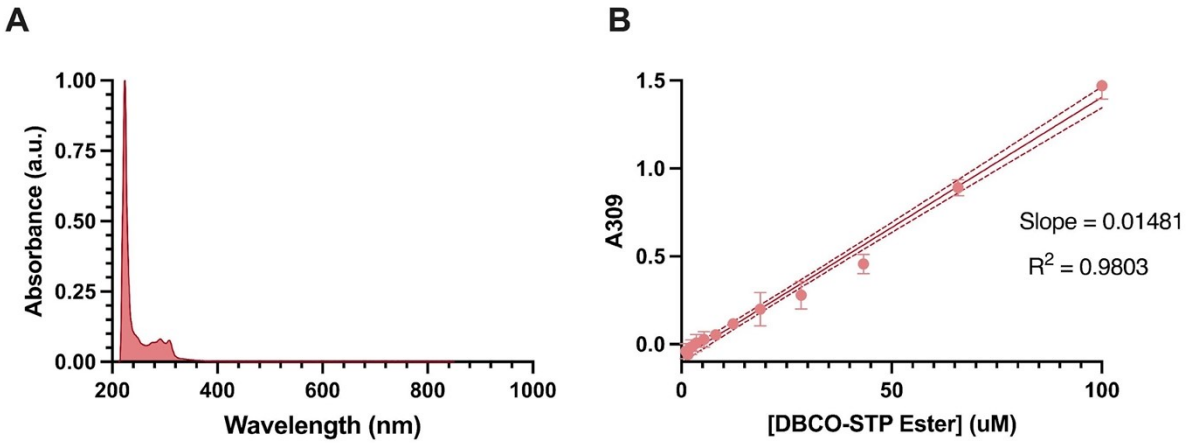

Figure S1: UV-Vis spectrum (A) and calibration curve (B) of DBCO-STP ester.
Data fit to a linear regression with two confidence bands surrounding the best-fit line (dashed lines). Error bars represent SD (n = 3).

ε_{309} = 14810 $M^{-1}\,cm^{-1}$

CF (A_{280}/A_{309}) = 0.849

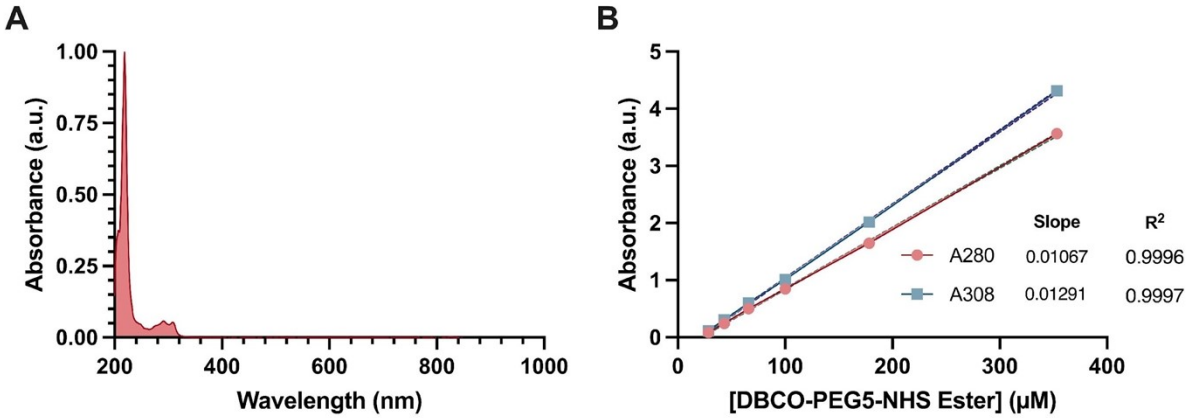

Figure S2: UV-Vis spectrum (A) and calibration curve (B) of DBCO-PEG5-NHS ester.
Data fit to a linear regression with two confidence bands surrounding the best-fit line (dashed lines). Error bars represent SD (n = 3).

ε_{308} = 12910 $M^{-1}\,cm^{-1}$

CF (A_{280}/A_{308}) = 0.812

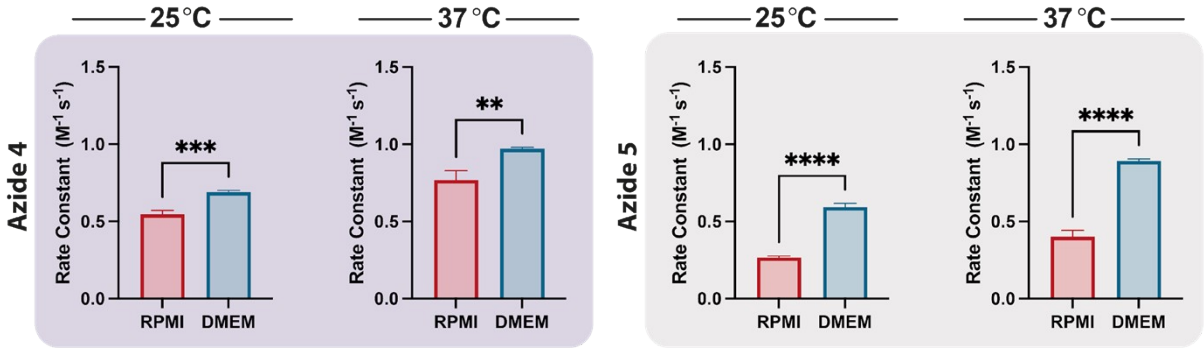

Figure S3: The effect of cell culture media on the second-order rate constants of SPAAC reaction.

*Reactions performed with 1-azido-1-deoxy-β-D-glucopyranoside (**4**) or 3-adizo-L-alanine HCl (**5**) in DMEM or RPMI at either 25 or 37 °C. Error bars represent SD (n = 3).*

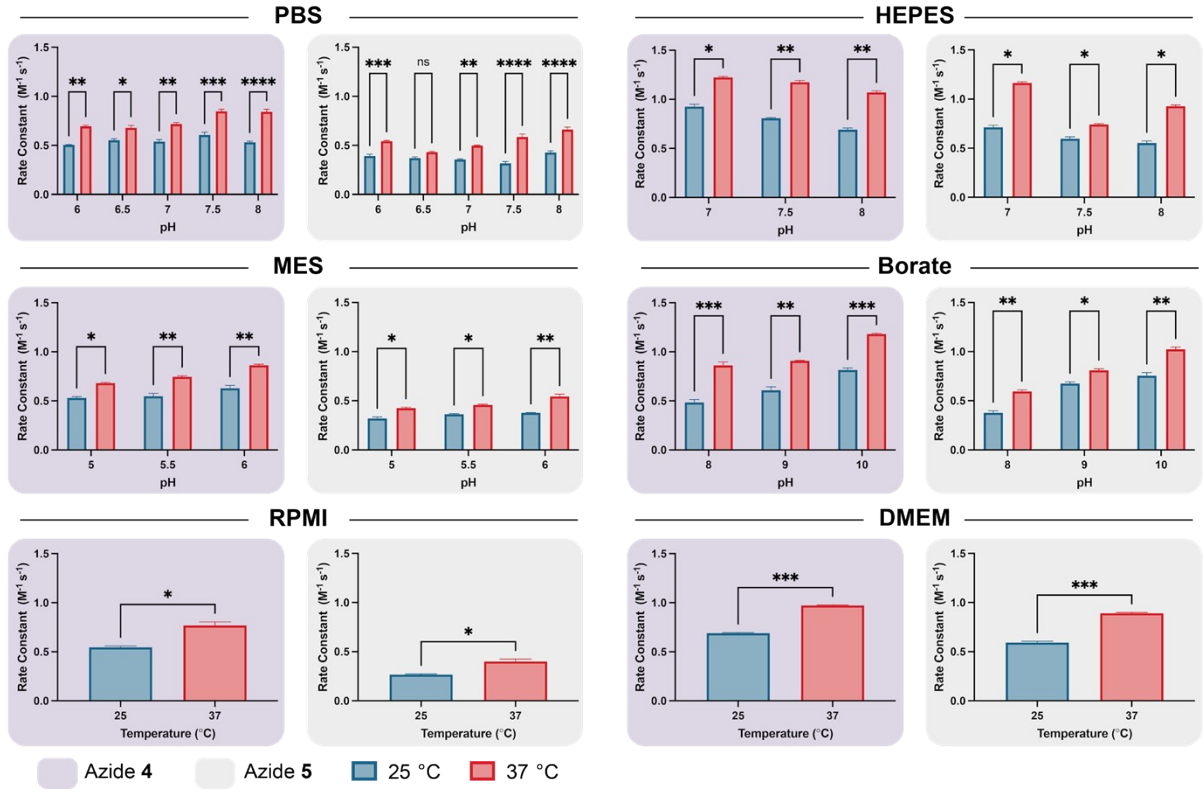

Figure S4: Effect of temperature on the second-order rate constant (k_2, [$M^{-1}s^{-1}$]) of SPAAC reactions.

*Reactions performed with sulfo DBCO-amine (**1**) and 1-azido-1-deoxy-β-D-glucopyranoside (**4**) or 3-adizo-L-alanine HCl (**5**) at 25 and 37 °C. Error bars represent SD (n = 3).*

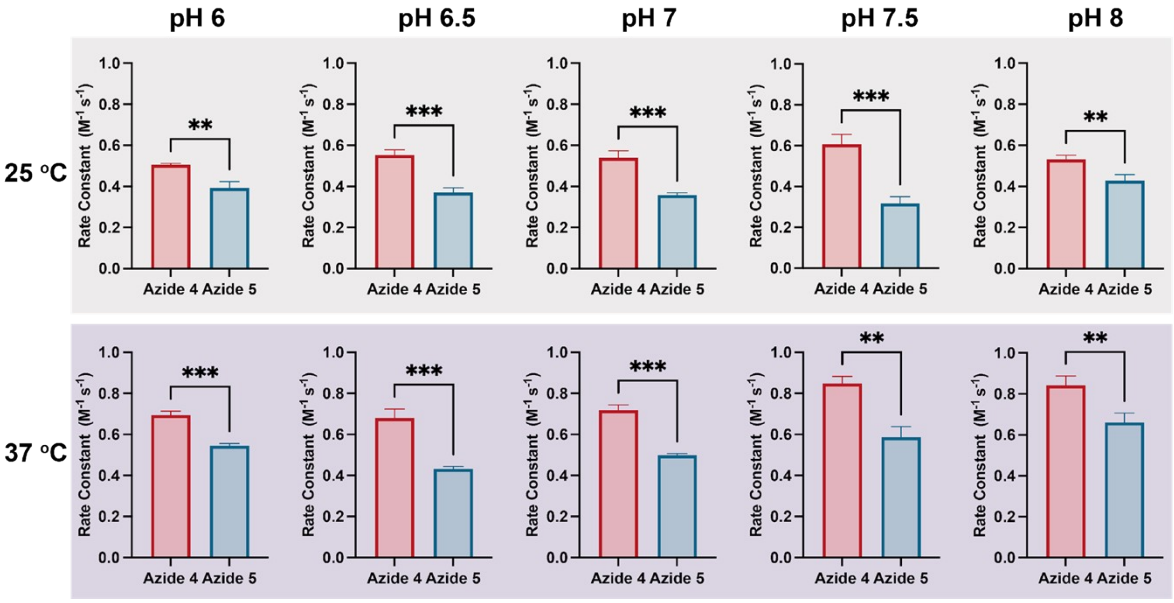

Figure S5: Effect of azide on SPAAC reaction rates in PBS.
Error bars represent SD, n = 3.

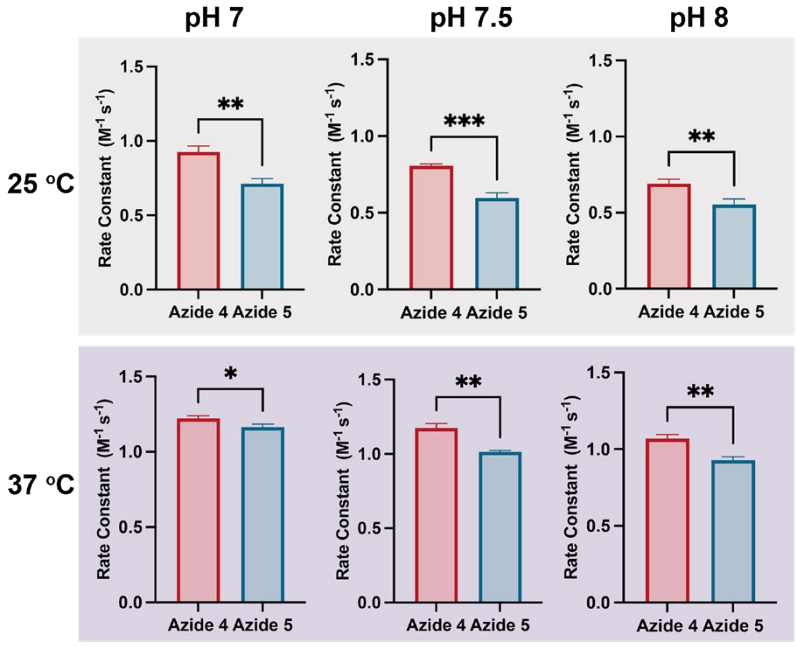

Figure S6: Effect of azide on SPAAC reaction rates in HEPES.
Error bars represent SD, n = 3.

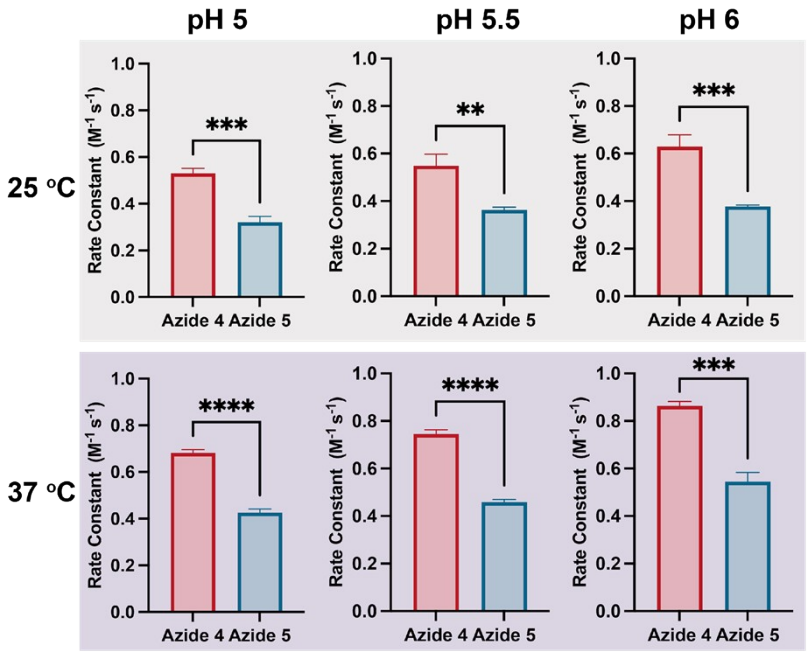

Figure S7: Effect of azide on SPAAC reaction rates in MES.
Error bars represent SD, n = 3.

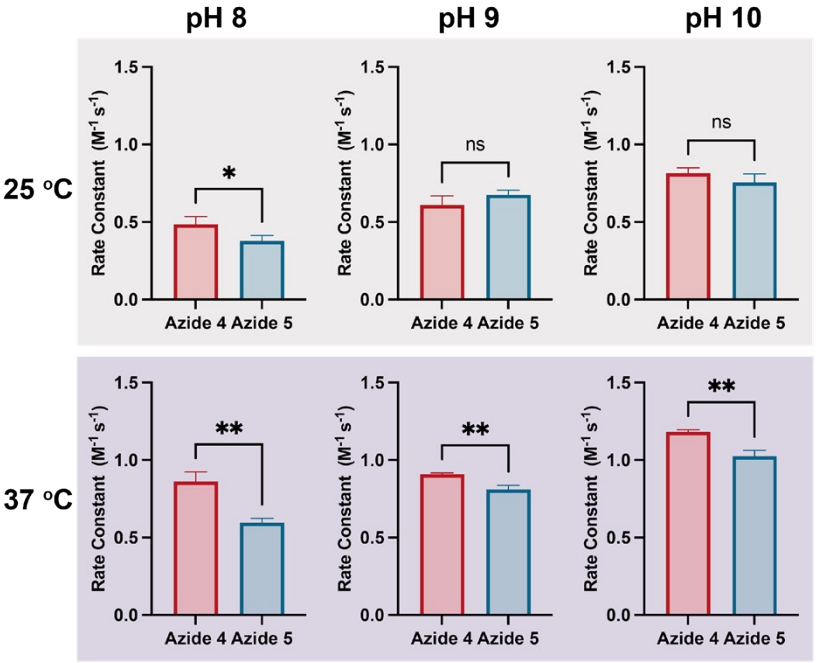

Figure S8: Effect of azide on SPAAC reaction rates in borate buffer.
Error bars represent SD, n = 3.

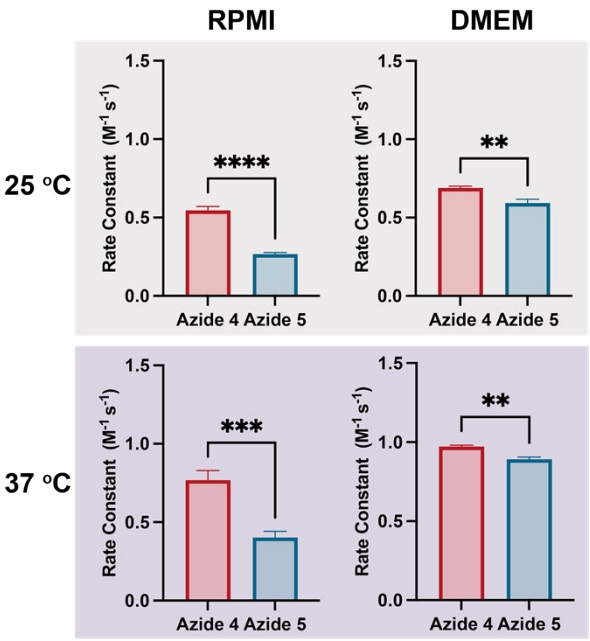

Figure S9: Effect of azide on SPAAC reaction rates in RPMI and DMEM media.

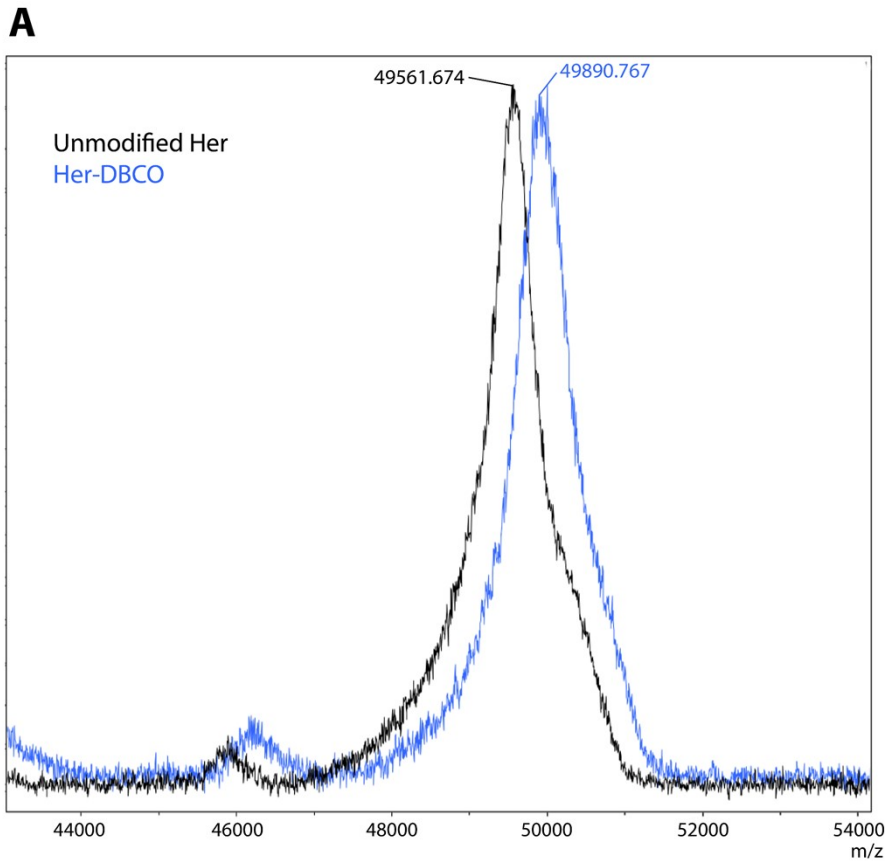

Figure S10: MALDI-TOF spectra of unmodified Herceptin (black) and Her-DBCO (blue). *Error bars represent SD, n = 3.*

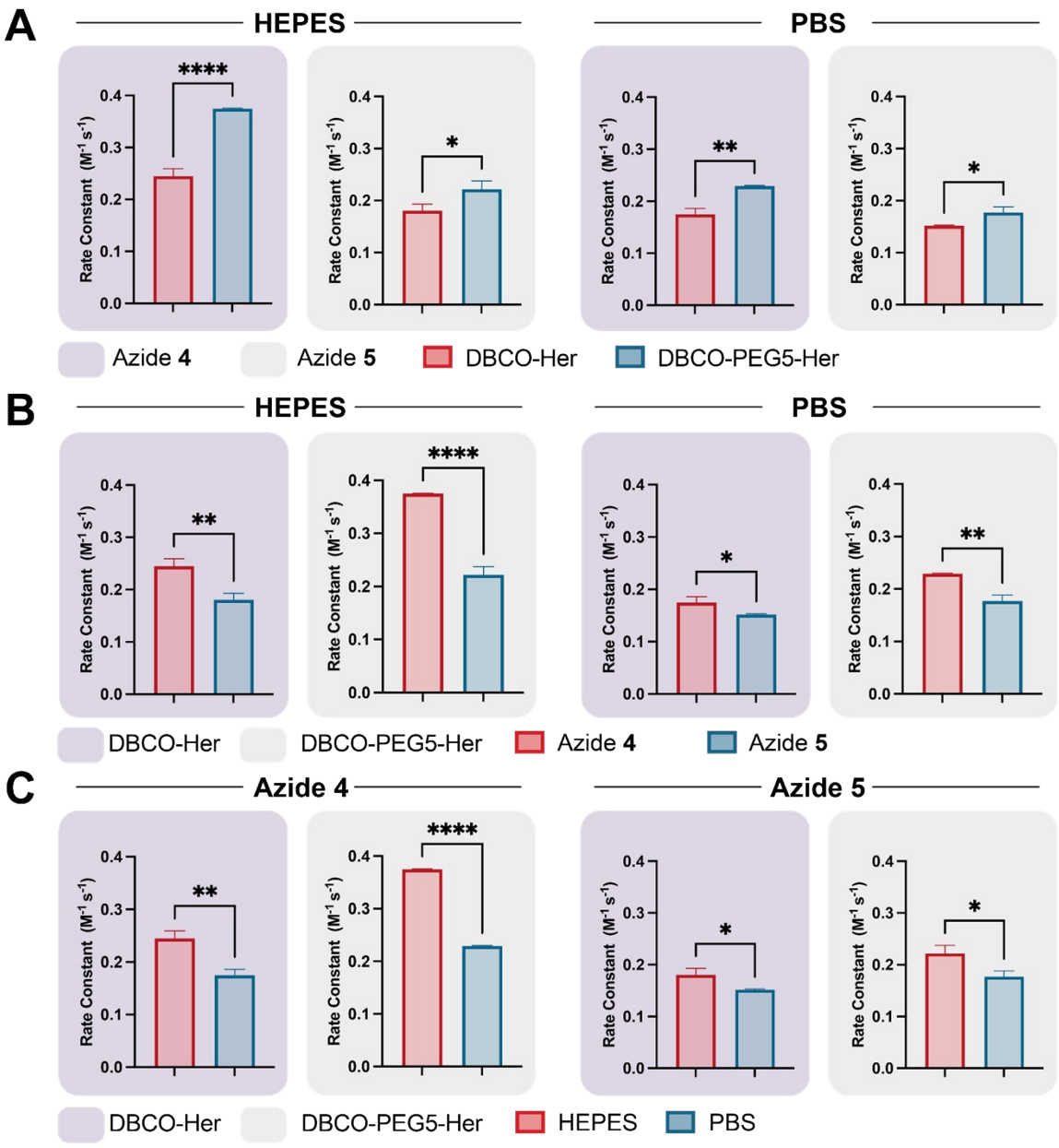

Fig. S11 Influence of selected reaction parameters on mAb-based SPAAC second-order rate constants.

A) Effect of PEG linker; B) Effect of azide; C) Effect of buffer. Reactions were performed with DBCO-Her (2) or DBCO-PEG5-Her (3) and 1-azido-1-deoxy--D-glucopyranoside (4) or 3-azido-L-alanine (5). Error bars represent SD (n = 3).

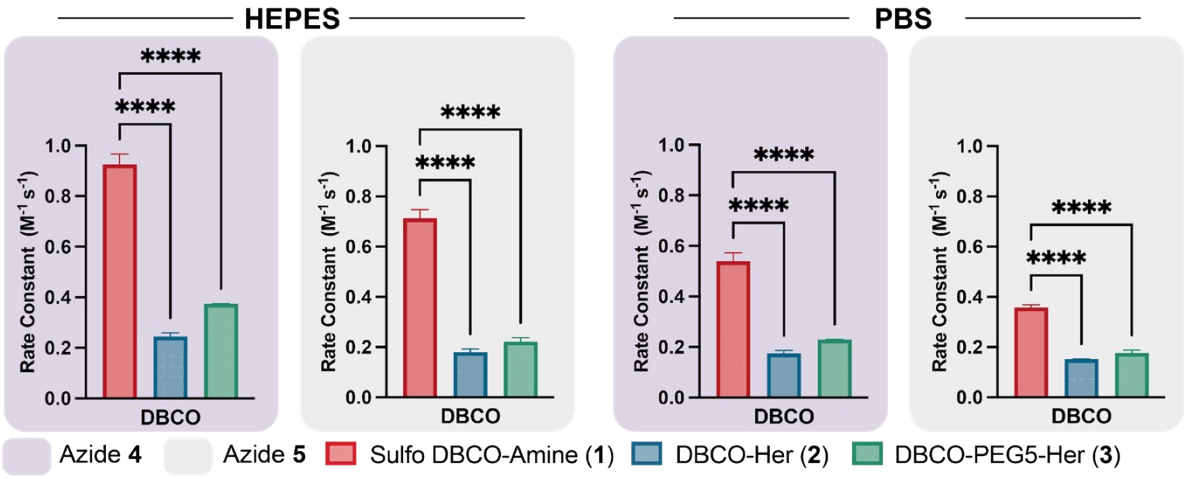

Figure S12: Second-order rate constant comparison for different DBCO reagents.
*Reactions performed with sulfo DBCO-amine (**1**), DBCO-Her (**2**), or DBCO-PEG5-Her (**3**) and 1-azido-1-deoxy-□-D-glucopyranoside (**4**) or 3-adizo-L-alanine HCl (**5**). Error bars represent SD (n = 3).*

Tables

	1 and **4**		**1** and **5**	
pH	**Rate constant at 25 °C ($M^{-1}s^{-1}$)**	**Rate constant at 37 °C ($M^{-1}s^{-1}$)**	**Rate constant at 25 °C ($M^{-1}s^{-1}$)**	**Rate constant at 37 °C ($M^{-1}s^{-1}$)**
		PBS		
6	0.50 ± 0.01	0.69 ± 0.02	0.39 ± 0.03	0.54 ± 0.01
6.5	0.55 ± 0.03	0.68 ± 0.04	0.37 ± 0.02	0.43 ± 0.01
7	0.54 ± 0.03	0.72 ± 0.03	0.36 ± 0.01	0.50 ± 0.01
7.5	0.61 ± 0.05	0.85 ± 0.03	0.32 ± 0.03	0.59 ± 0.05
8	0.53 ± 0.02	0.84 ± 0.04	0.43 ± 0.03	0.66 ± 0.04
		HEPES		
7	0.93 ± 0.04	1.22 ± 0.02	0.71 ± 0.03	1.16 ± 0.02
7.5	0.81 ± 0.01	1.17 ± 0.03	0.60 ± 0.03	1.01 ± 0.01
8	0.69 ± 0.03	1.07 ± 0.03	0.55 ± 0.04	0.93 ± 0.02
		MES		
5	0.53 ± 0.02	0.68 ± 0.01	0.32 ± 0.03	0.43 ± 0.02
5.5	0.54 ± 0.05	0.75 ± 0.02	0.36 ± 0.01	0.46 ± 0.01
6	0.63 ± 0.05	0.86 ± 0.02	0.38 ± 0.01	0.54 ± 0.04
		Borate		
8	0.48 ± 0.05	0.86 ± 0.06	0.38 ± 0.03	0.60 ± 0.03
9	0.61 ± 0.06	0.91 ± 0.01	0.68 ± 0.03	0.81 ± 0.03
10	0.82 ± 0.03	1.18 ± 0.01	0.76 ± 0.05	1.02 ± 0.04
		RPMI		
7.5	0.55 ± 0.02	0.77 ± 0.06	0.27 ± 0.01	0.40 ± 0.04
		DMEM		
7.5	0.69 ± 0.01	0.97 ± 0.01	0.59 ± 0.02	0.89 ± 0.01

Table S1: Second-order rate constants (k_2 ± SD [$M^{-1}s^{-1}$]) of SPAAC reactions in various conditions with sulfo DBCO-amine (1).

Reactions performed in different buffers and pH values. ***4*** *= 1-azido-1-deoxy-□-D-glucopyranoside,* ***5*** *= 3-adizo-L-alanine HCl. Reactions performed in triplicate and reported as mean ± SD.*

Tukey's Multiple Comparisons Test	Adjusted *P*-value	Summary
PBS vs. HEPES	<0.0001	****
PBS vs. MES	0.9652	ns
PBS vs. Borate	<0.0001	****
PBS vs. RPMI	0.1475	ns
PBS vs. DMEM	0.0051	**
HEPES vs. MES	<0.0001	****
HEPES vs. Borate	0.7014	ns
HEPES vs. RPMI	<0.0001	****
HEPES vs. DMEM	<0.0001	****
MES vs. Borate	<0.0001	****
MES vs. RPMI	0.0409	*
MES vs. DMEM	0.0189	*
Borate vs. RPMI	<0.0001	****
Borate vs. DMEM	<0.0001	****
RPMI vs. DMEM	<0.0001	****

Table S2: One-way ANOVA with Tukey's post-hoc multiple comparisons tests to compare SPAAC rates in buffers.

	1 and **4**		**1** and **5**	
	25 °C	**37 °C**	**25 °C**	**37 °C**
		PBS		
Slope	0.0217	0.0930	0.0037	0.0775
R^2	0.1359	0.6929	0.0037	0.4497
P-value	0.1763 (ns)	0.0001	0.8293 (ns)	0.0062
		HEPES		
Slope	-0.2352	-0.1517	-0.1596	-0.2361
R^2	0.9398	0.8624	0.7934	0.9550
P-value	<0.0001	0.0003	0.0013	<0.0001
		MES		
Slope	0.0990	0.1817	0.0579	0.1193
R^2	0.5367	0.9391	0.7036	0.8022
P-value	0.0248	<0.0001	0.0047	0.0011
		Borate		
Slope	0.1658	0.1602	0.1890	0.2143
R^2	0.9043	0.8204	0.8647	0.9797
P-value	<0.0001	0.0008	0.0003	<0.0001

Table S3: Tabular results of simple linear regression of the second-order rate constant (k_2, [$M^{-1}s^{-1}$]) as pH increases for each buffer.

*Reactions performed with sulfo DBCO-amine (**1**) and 1-azido-1-deoxy-□-D-glucopyranoside (**4**) or 3-adizo-L-alanine HCl (**5**) at 25 and 37 ℃. ns = not significant. R^2 values represent goodness-of-fit of the simple linear regression. P-values test that the slope is significantly different than zero.*