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Plasma induced acceleration and selectivity in strain-promoted azide–alkyne cycloadditions†

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Strain-promoted azide–alkyne cycloaddition (SPAAC) is an important member of the bioorthogonal reaction family. Over the past decade, much work has been dedicated to the generation of new strained alkynes with improved reactivity. While kinetics studies of SPAAC are often conducted in organic solvents, buffered solutions or mixtures, these media do not reflect the complexity of *in vivo* systems. In this work, we show that performing SPAAC in human plasma leads to intriguing kinetics and selectivity effects. In particular, we observed that reactions in plasma could be accelerated up to 70-fold compared to those in methanol, and that selective couplings between a pair of reagents could be possible in competition experiments. These findings highlight the value of evaluating bioorthogonal reactions in such a complex medium, especially when *in vivo* applications are planned, as unsuspected behaviour can be observed, disrupting the usual rules governing the reactivity in simple solvent systems.

Introduction

Bioorthogonal reactions can be described as chemical reactions occurring in a biological environment without interfering with any of its processes or materials. Initially coined by Carolyn R. Bertozzi in the early 2000s, bioorthogonal chemistry has since benefited from many improvements in terms of reaction selectivity and kinetics thanks to the development of more soluble and stable reagents with reduced toxicities.[1–9] Strain-promoted azide–alkyne cycloaddition (SPAAC) is a striking example of such an achievement: reported for the first time in the context of bioorthogonal chemistry in 2004, it is now considered as an indispensable reaction of the chemical biology toolbox, due to the stability of the resulting triazole products, along with its simplicity of operation, low toxicity and fast kinetic rates.[6] The latter characteristic is a crucial parameter to take into account for *in vivo* applications, as the reaction has to be rapid in order to occur at low concentrations before reactants are metabolized or excreted. Major efforts have thus been dedicated to optimizing SPAAC kinetic rates *via* the development of more reactive alkynes through the modulation of stereoelectronic parameters.[10–14] In this race for the ideal bioorthogonal reagent, the effect of the surrounding biological environment in which the reaction takes place has been somehow overlooked. Even though it is well known that solvent effects strongly influence cycloaddition rates,[15] most studies have been conducted in organic–aqueous solvent mixtures, these systems being often considered as the representative of more complex biological media. Interestingly however, the group of Carolyn R. Bertozzi reported the unexpected reactivity of several alkyne probes when using SPAAC to label azide-functionalized glycans in living mice.[16] Despite the comparable labelling efficiencies *in vitro*, in coherence with similar second-order rate constants of the parent cyclooctynes in model reactions,[17–19] several alkyne probes gave markedly different results when tested *in vivo*, suggesting that the *chemical structure* of the probe itself was a key parameter of its *in vivo* reactivity. In the same vein, Robillard and co-workers described the use of SPAAC reactions for the modification of azide-containing antibodies in live mice and showed that serum proteins – and especially albumin – had a negative impact on the reaction kinetics, due to their strong interactions with alkynes, making the latter less available to react with the antibodies' azide groups.[20] Altogether, these results support the hypothesis that the outcome of bioorthogonal reactions *in vivo* cannot be predicted solely based on the parent alkyne's reactivity in model reactions conducted in simple solvent systems. As more complex parameters may play a substantial role, new *in vitro* models are thus needed to mimic this environment in order to decipher the underlying rules governing the fate of bioorthogonal reactions in complex media.

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Results and discussion

Building on a previous study in which we reported the *in vivo* neutralization of the azido derivative **1** of the anticoagulant warfarin by a SPAAC reaction with bicyclononyne (BCN) **3**,[15] we started by evaluating human plasma as a surrogate for *in vivo* models. It became quickly apparent that rate constants were extremely similar between *in vivo* and plasma media, and in sharp contrast to those measured with the model azide **2** in both methanol and phosphate buffered saline (PBS) solutions (Fig. 1 and S2†). While such strong discrepancies underlined a major influence of the medium on SPAAC kinetics, the fact that we were comparing two different systems (**1** → **4** in plasma and in mice *vs.* **2** → **5** in PBS and methanol) prevented us from drawing this definitive conclusion.

In order to probe the influence of plasma on the outcome of SPAAC reactions, we decided to perform a systematic study by measuring the cross reactivity of alkynes/azides pairs under standardized conditions and determining their corresponding second order kinetic rate constants. We designed a coherent series of BCN derivatives, all synthesized from the simple BCN alcohol **6**: one bearing an apolar dioxole group (**7**) and three equipped with polar moieties, displaying either a permanent charge at physiological pH (**8** and **9**) or possessing a short and neutral oligoethyleneglycol (OEG) chain (**3**). Each of these BCN probes was paired with a counterpart azide equipped with similar functional groups (**2**, **10**–**12**). These moieties were chosen with the aim of modulating the biophysical properties of the reagents without affecting their intrinsic chemical reactivity. Each pair was then reacted under equimolar conditions at a concentration of 0.1 mM in human plasma. This standard concentration was chosen since it was consistent with the maximum plasmatic concentration of PEG **3** measured after sub-cutaneous injection (52 mg kg^{-1}) in mice.[15] In methanol, the concentration had to be increased to 5 mM in order to start seeing the product formation within the two hours timeframe of a classical experiment. In each case, 1,7-dihydroxynaphthalene was used as an internal standard in order to improve the precision of our analytical method, and the reactions were monitored by HPLC at various time points, following both the azide disappearance and triazole product formation (see the ESI†). As all BCN and azide compounds evaluated in these experiments were perfectly stable in plasma and since the expected triazole was the only product formed in all reactions, the second-order kinetic constant of the different SPAAC reactions was calculated by plotting the evolution of the inverse of the concentration of the azide component as a function of time according to the following equation:

$$1/[A] = 1/[A]_0 + kt$$

where $[A]_0$ is the initial molar concentration of the azide reactant; [A], the molar concentration of the azide reactant at a given time; t, the time in seconds; and k, the second-order kinetic constant expressed in M^{-1} s^{-1} (see the ESI† for details about calculations).

We found that SPAAC kinetics were quite slow in methanol ($\leq$ 0.57 M^{-1} s^{-1}) and with little variations between each BCN/azide pair evaluated (σ ~0.17 M^{-1} s^{-1}), clearly suggesting that the reactant structure had little to no influence on the outcome of SPAAC reactions in organic solvents (Fig. 2). In plasma however, reactions were found to be greatly accelerated compared to methanol, up to 70-fold with second-order kinetic constant values culminating at ~19 M^{-1} s^{-1}. In addition, striking differences in reactivity were found between the different reactant pairs depending on their structure and skeleton (σ ~4.73 M^{-1} s^{-1}). One notable exception concerned carboxylate-bearing azide **12**, whose reactivity was barely impacted by either the BCN reagent or the medium composition. At this stage, it is important to reiterate that reactions were fifty times more concentrated in methanol than in plasma (5 mM *vs.* 0.1 mM), and differences in conversion rates between the two media were logically even more pronounced when reactions were conducted at 0.1 mM in both the cases (see the ESI†).

Lastly, comparison between the kinetics in PBS and methanol showed that while reactions between azide **11** and all BCN probes were accelerated in water – a feature previously reported in the literature – this was to a much lesser extent than in plasma, highlighting that the aqueous nature of the reaction medium cannot be the sole explanation for the effects observed (see the ESI†). Altogether, these data demonstrate that the reactivity of a strained alkyne or an azide in plasma can be strongly modulated by the chemical structure of the moieties appended to it. Consequently, by performing SPAAC in plasma instead of classical solvent systems, it should be possible in theory to discriminate between two azides or two BCN and attain unprecedented selectivity.

We thus selected BCN-azide pairs **3**–**11** and **3**–**12**, whose corresponding SPAAC showed similar kinetic rates in metha-

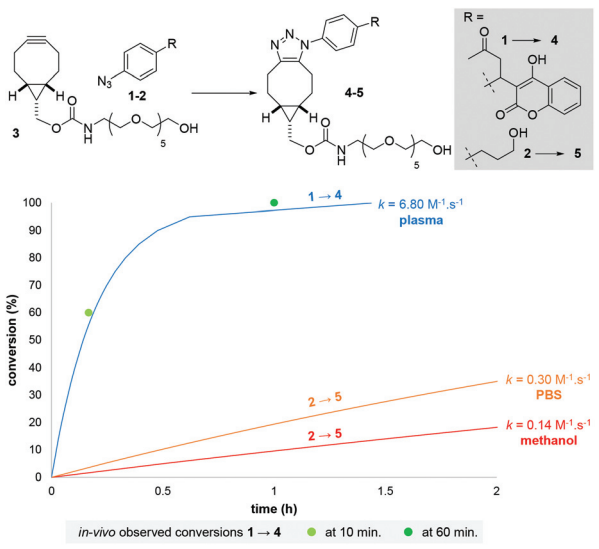

Fig. 1 Differences in kinetics between strain-promoted azide–alkyne cycloadditions run either in classical solvent systems or in plasma. The green dots represent the conversion observed in vivo.

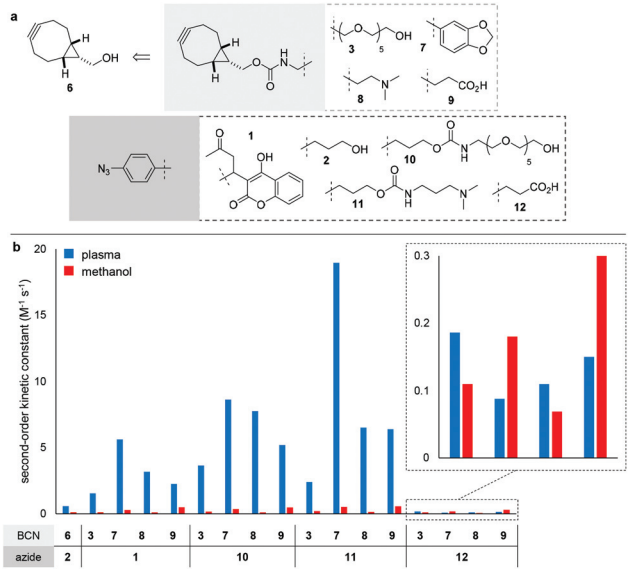

Fig. 2 Influence of azide and cyclooctyne structures on the rate of SPAAC reactions; (a) structures of the different BCN and azide derivatives utilized in the study; (b) representation of the different second-order kinetic constants measured in plasma (100 μM) and in methanol (5 mM) for the SPAAC reactions between different BCN–azide pairs. These values represent the average of measurements made in triplicate (n = 3).

nol (0.21 M^{-1} s^{-1} and 0.11 M^{-1} s^{-1}, respectively) but substantial differences in plasma (2.39 M^{-1} s^{-1} and 0.19 M^{-1} s^{-1}) and performed competition experiments. As expected, both azides were found to be equally reactive at 0.1 mM in methanol, leading to an equimolar mixture of triazoles **13** and **14** (Fig. 3A). As anticipated also at this concentration and in this solvent, less than 10% conversion was observed after 60 minutes. In plasma however, azide **11** predominantly and quickly reacted, leading to ~40% conversion to the expected triazole product **13** after 60 minutes, while azide **12** did not participate in any visible reaction (blue line and red line, respectively; Fig. 3B). This illustrates that the selectivity between two nearly identical SPAAC can be attained just by switching from a classical solvent to plasma.

To evaluate the potential applications of such behaviour in the context of *in vivo* chemistry, where SPAAC is usually conducted with an excess of strained alkyne, the concentration of BCN **3** was increased to 0.5 mM while keeping the same concentrations in azides **11** and **12** (*i.e.* 0.1 mM), now making them the limiting reactants. As expected, faster reactions were observed in both methanol and plasma (Fig. 3C and D). Despite this acceleration, the same trend as before was still observed. No differences between **11** and **12** were noticed in methanol at 0.5 mM and poor conversion was detected for both substrates, while an even better level of selectivity between azides **11** and **12** was found in plasma: while carboxylate derivative **12** gave less than 20% conversion to triazole **14**, the ammonium derivative **11** gave almost complete conversion to **13** after 60 minutes.

To understand better the underlying effects at stake in these reactions, we conducted plasma protein-binding assays to determine the fraction of free azides and BCNs in plasma. Utilizing the equilibrium dialysis method (see the ESI† for detailed procedures), which had previously allowed us to determine the percentage of warfarin azide **1** bound to plasma proteins to be 94%,[15] we found sharp variations between our set of compounds (Fig. 4). Binding values of azides were shown to range from 15% for compound **2** to 98% for **1**. A comparable variation was also observed for BCN, going from 26% for both **3** and **9** to almost 99% for compound **7**. This plasma–protein binding affinity did not seem to be directly correlated with the functional groups equipping the SPAAC reagents: primary alcohol-containing azide **2** and BCN **6** gave completely opposite values (15% and 91%, respectively), similar to what was observed with OEG-functionalized BCN **3** (26%) and azide **10** (79%). When comparing binding values and second order kinetic constants in an attempt to find a correlation between the two sets of values, we observed that the most bound azides (*i.e.* **1**, **10** and **11**) systematically gave the highest SPAAC kinetics, while the less bound azides **2** and **12** led to mediocre rate constants. In addition, for a given azide, SPAAC kinetics appeared to be closely related to and modulated by the percentage of protein-bound BCNs, with compound **7** giving always the best results. These findings relate well to the previously mentioned study from the Robillard group, who also reported that serum proteins strongly interact with alkynes.[20] While in the latter case this proved to be detrimental to SPAAC reactions with azide groups borne by bulky immunoconjugates, a similar effect might be beneficial for reactions between compounds of lower molecular weights. Even though other factors might also be at stake, such as the compound's hydrophobicity or flexibility, the binding of reagents to surrounding proteins seems to have a major influence on the acceleration of SPAAC in plasma. Although counterintuitive, a local increase of concentration at the surface of a plasma protein could facilitate the interactions between azides and BCN in such a dilute medium and result in the acceleration of SPAAC.

To validate this hypothesis, we reacted our previously described model system – BCN **3** and azide **1** –[15] in PBS in the presence of increasing amounts of human serum albumin (HSA, see the ESI† for more details), the most abundant protein in human plasma.[21] Gratifyingly, the second-order kinetic constant determined at plasma-like concentrations of HSA in PBS (*i.e.* 50 g L^{-1}) was found to be in agreement with that measured in human plasma (1.66 M^{-1} s^{-1} *vs.* 1.55 $M^{-1}s^{-1}$, respectively). While this observation tends to support the hypothesis that protein binding is responsible for the acceleration of SPAAC in human plasma, we also found that the highest rate acceleration was observed in relatively diluted HSA solutions (3.33 M^{-1} s^{-1} at 2.5 mg mL^{-1} of HSA in PBS). This shows that plasma proteins and their concentrations exert a strong influence on the kinetics of SPAAC. Understanding better the factors governing this peculiar behaviour and the role of these "participating spectators" in biological media could open up new directions in chemical biology.

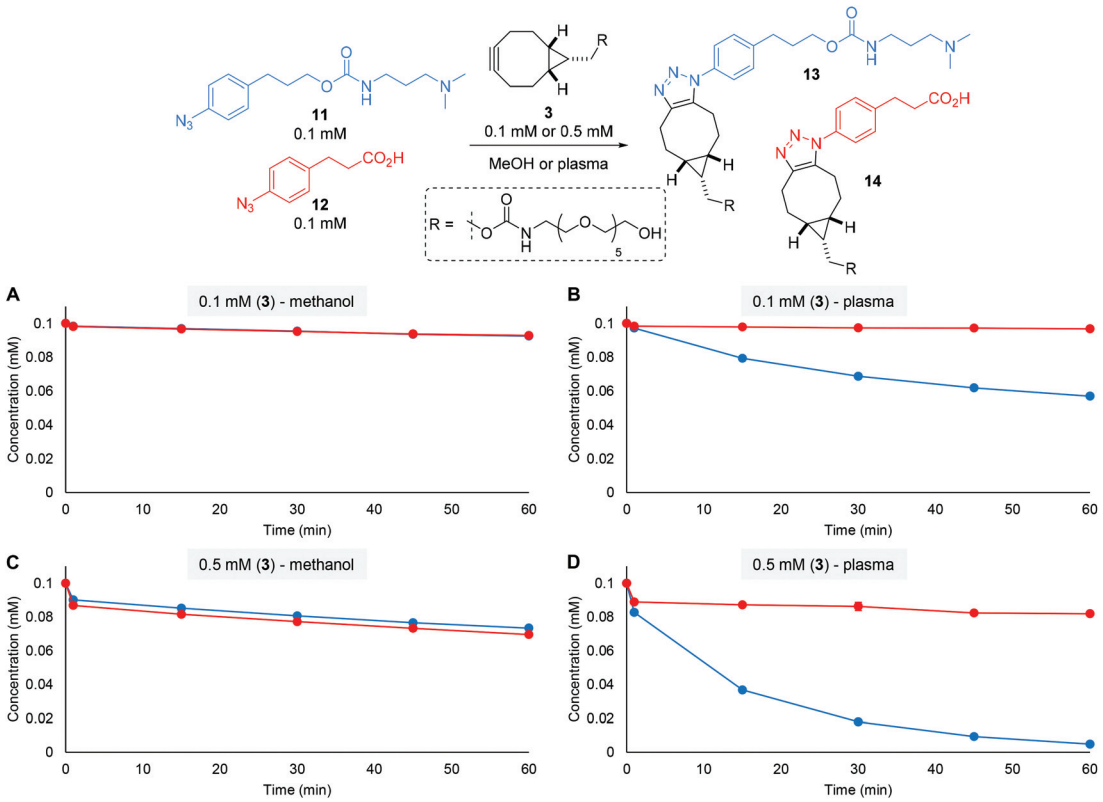

Fig. 3 Competition experiments between azides **11** and **12** for reaction with BCN **3** *via* SPAAC and the influence of the medium composition and reactant concentration on the observed conversion. Measurements were made in triplicate (n = 3).

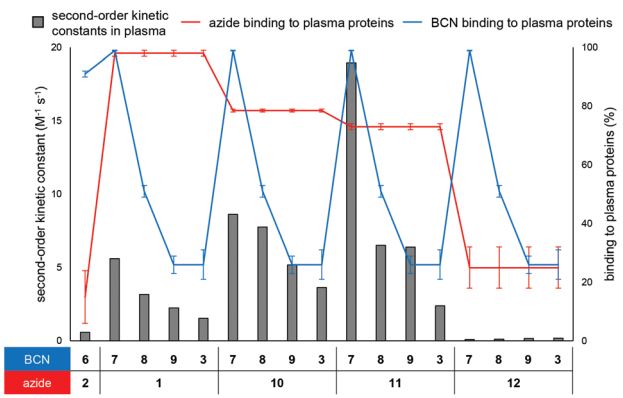

Fig. 4 Plasma protein-binding values for all BCN and azide compounds evaluated (curves) plotted together with the previously determined second order kinetic constants of SPAAC reactions in plasma (histograms). Measurements were made in triplicate (n = 3).

Conclusions

In conclusion, we showed the rate enhancement of various SPAAC reactions conducted in plasma compared to methanol and PBS. Functional groups borne by different BCN and azide moieties were found to have a profound impact on the reactions' kinetic constants. We took advantage of this effect to successfully conduct competition experiments, where only one of two azide compounds reacted with a given BCN. To the best of our knowledge, this is the first report of an *in situ* selectivity in plasma between two identical bioorthogonal reactions, only caused by the chemical structure of the reactants. Exploring the interactions between the biological medium and the reactive partners of the SPAAC reaction and including this medium as an influential reaction partner should be taken into account when developing or optimizing bioorthogonal reactions. Such phenomena provide unexpected perspectives in the development of effective orthogonal pairs based on a single reactive core and expand the *in vivo* chemistry toolbox while alleviating the need for the production of new families of strained alkynes.

Author contributions

DW took care of investigation, methodology, validation, visualisation and writing of the original draft of this manuscript. ED took care of methodology and validation. MR and SU took care of data curation & early work and investigation. MM and JS took care of methodology. WK took care of conceptualisation, funding acquisition, resources, supervision and reviewing & editing of the final manuscript. GC took care of funding acquisition, project administration, supervision, visualisation, writing of the original draft and reviewing & editing of the final manuscript. AW took care of conceptualisation, funding

acquisition, project administration, resources, supervision and reviewing & editing of the final manuscript.

Conflicts of interest

There are no conflicts to declare.

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