ChemPubSoc Europe

DOI: 10.1002/open.201402104

Open Access ChemistryOPEN Full Papers

Quantum Chemical Calculations and Experimental Validation of the Photoclick Reaction for Fluorescent Labeling of the 5′ cap of Eukaryotic mRNAs

Daniela Stummer,[a, b] Carmen Herrmann,*[c] and Andrea Rentmeister*[a, b]

Bioorthogonal click reactions are powerful tools to specifically label biomolecules in living cells. Considerable progress has been made in site-specific labeling of proteins and glycans in complex biological systems, but equivalent methods for mRNAs are rare. We present a chemo-enzymatic approach to label the 5′ cap of eukaryotic mRNAs using a bioorthogonal photoclick reaction. Herein, the *N*7-methylated guanosine of the 5′ cap is enzymatically equipped with an allyl group using a variant of the trimethylguanosine synthase 2 from *Giardia lamblia* (GlaTgs2). To elucidate whether the resulting N^2-modified 5′ cap is a suitable dipolarophile for photoclick reactions, we used Kohn–Sham density functional theory (KS-DFT) and calculated the HOMO and LUMO energies of this molecule and nitrile imines. Our in silico studies suggested that combining enzymatic allylation of the cap with subsequent labeling in a photoclick reaction was feasible. This could be experimentally validated. Our approach generates a turn-on fluorophore site-specifically at the 5′ cap and therefore presents an important step towards labeling of eukaryotic mRNAs in a bioorthogonal manner.

Introduction

Establishing methods to label biomacromolecules both in vitro and in vivo is of tremendous interest. Numerous approaches to mark proteins or glycans on or in cells have been described.[1–4] Many of these labeling strategies combine enzymatic introduction of a reactive handle into the biomolecule with subsequent chemical modification by a suitable click reaction.[5]

Labeling of biomolecules in living cells is considered particularly rewarding because novel imaging techniques allow live-cell analysis and provide insights into the dynamic cellular processes that these biomolecules are involved in. Regarding mRNAs, studying their trafficking and subcellular localization may provide insights into novel regulatory mechanisms.[6]

Consequently, within the last years, several approaches were introduced and refined to visualize mRNAs in living cells. Simple complementary oligonucleotides were used as probes initially,[7] before modified and thus more stable ones were developed.[8] The introduction of molecular beacons was an important improvement in live-cell RNA imaging, because a signal is generated upon binding of the probe to its target, which decreases the background signal from unbound probes—one of the main challenges in live-cell labeling.[9,10]

A different strategy is based on RNA motifs (like the stem loop from the phage MS2) that bind tightly and specifically to natural RNA-binding proteins. The RNA of interest is extended by several of these motifs and detected by the respective RNA-binding protein fused to a fluorescent protein.[11,12] These approaches circumvent delivery problems associated with labeled oligonucleotides as probes and have provided valuable insights into RNA localization and dynamics in different cell types and even organisms.[11–15] Fusion of an in vitro selected motif, namely a "turn-on" aptamer, to the RNA of interest was recently realized and may find broad application, but still requires to extend the RNA of interest by a non-natural tag.[16,17]

An alternative system based on the RNA-binding protein Pumilio and split-GFP (green fluorescent protein) was established for RNA detection in vitro and in cells.[18–21] Since Pumilio binds to RNA sequences specifically, the RNA of interest does not have to be extended by a tag, which is a major advantage. The bipartite or tripartite split-GFP becomes fluorescent only upon correctly positioned binding of the Pumilio proteins.[18,20,22,23]

[a] *Dr. D. Stummer,[+] Prof. A. Rentmeister[+]*
Institute of Biochemistry, Westfälische Wilhelms-Universität Münster,
Wilhelm-Klemm-Straße 2, 48149 Münster (Germany)
E-mail: a.rentmeister@uni-muenster.de

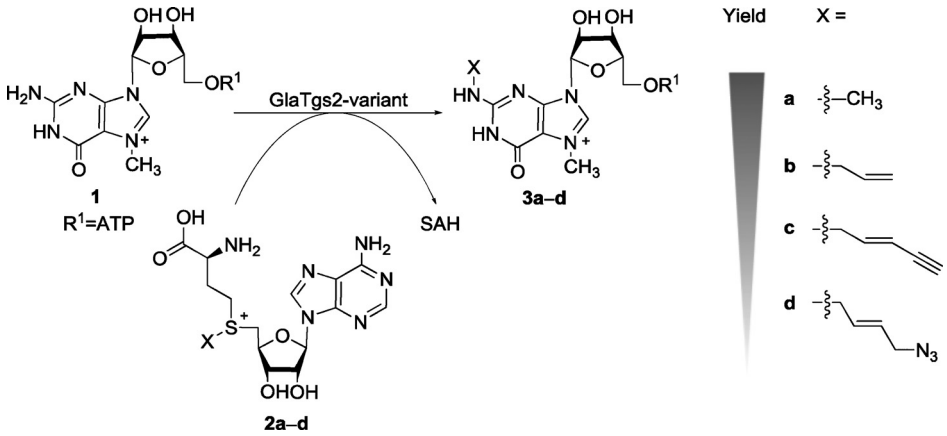
[b] *Dr. D. Stummer,[+] Prof. A. Rentmeister[+]*
Cells-in-Motion Cluster of Excellence (EXC 1003-CiM)
Westfälische Wilhelms-Universität Münster
Wilhelm-Klemm-Straße 2, 48149 Münster (Germany)

[c] *Prof. C. Herrmann[+]*
Institute of Inorganic Chemistry, University of Hamburg
Martin-Luther-King-Platz 6, 20146 Hamburg (Germany)
E-mail: carmen.herrmann@chemie.uni-hamburg.de

[+] *D. S., C. H., and A. R. designed the research. D. S. performed the experiments. C. H. performed the calculations. All authors analyzed and discussed the results. All authors wrote the manuscript.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/open.201402104.

© 2014 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.



Scheme 1. Possible modifications of the mRNA cap catalyzed by the GlaTgs2 variant. The GlaTgs2 variant can transfer several click reactive handles to the mRNA cap (R^1 = pppA) making it amenable to CuAAC, SPAAC, and thiol-ene click reactions. The yield of enzymatic conversions decreases with increasing size of the transferred moiety (X).

Beyond methods based on hybridization or binding, covalent modification of biomolecules, including RNA, has received considerable attention. Recent developments in the field of bioorthogonal chemistry have yielded a toolbox of reactions for labeling biomolecules even in complex environments.[2,24–27] The premise for these strategies is to equip the biomolecule of choice with an appropriate bioorthogonal group. In the field of RNA, enzymatic modification—particularly using RNA methyltransferases and cosubstrate analogs—has been very successful to selectively furnish RNA with a bioorthogonal group. In a second (chemical and ideally bioorthogonal) reaction, a reporter molecule becomes covalently linked to the modified target RNA. Although bioorthogonal reactions have been successfully used to convert modified RNA in cells, chemo-enzymatic approaches have so far only been used in vitro or in fixed cells to label DNA or RNA.[24,25,28–34]

In the frequently used copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), a Cu^I-activated terminal alkyne reacts with an azide to form a triazol.[35,36] Although CuAAC has been used successfully to label RNA in vitro as well as in fixed cells,[24,25,29,37] its application inside living cells seems to be limited due to the toxicity of copper(I).[38] Problems arising from copper are circumvented in the strain-promoted azide–alkyne cycloaddition (SPAAC) of cyclooctynes with azides, which form triazoles without requiring a catalyst.[27,37] However, application of SPAAC can be compromised in living cells due to cross reactivity of cyclooctyne derivatives and cellular thiols.[39] Although azides and alkynes are currently the most widely used bioorthogonal groups, alternatives have been explored, and some of them have attractive characteristics. The inverse-electron-demand Diels–Alder reaction between strained alkenes and tetrazines is biocompatible and was used for RNA labeling of chemically or enzymatically synthesized RNA containing appropriate dienophiles.[33,34]

We previously described a variant of trimethylguanosine synthase 2 from *Giardia lamblia* (GlaTgs2) with improved activity on analogs of the cosubstrate *S*-adenosyl-L-methionine that can be used to transfer alkene, alkyne, or azido groups to the 5′ cap of RNA.[32,33] We could show that enzymatic introduction of alkene, alkyne, or azido moieties at N^2 gives access to further derivatization of the cap in CuAAC, thiol-ene, or SPAAC reactions.[30,31] The yield of the enzymatic step currently depends on the cosubstrate and decreases with larger substituents (Scheme 1).

The conversion using 5′-[(*R*/*S*)(3*S*)-3-amino-3-carboxypropyl]-prop-2-enylsulfonio]-5′-deoxyadenosine (AdoPropen, **2b**) as cosubstrate is particularly efficient, yielding up to 90% conversion of the cap to *P1*-adenosine(5′)-*P3*-[N^2-prop-2-enyl,7-methylguanosine(5′)] triphosphate (N^2-allyl-modified cap **3b**). Conversion of **3b** in a thiol-ene reaction was successful in aqueous buffer but not compatible with cellular lysate, which is typically rich in thiols. We were therefore highly interested in establishing a bioorthogonal reaction that allows us to fluorescently label N^2-allyl-modified cap **3b**.

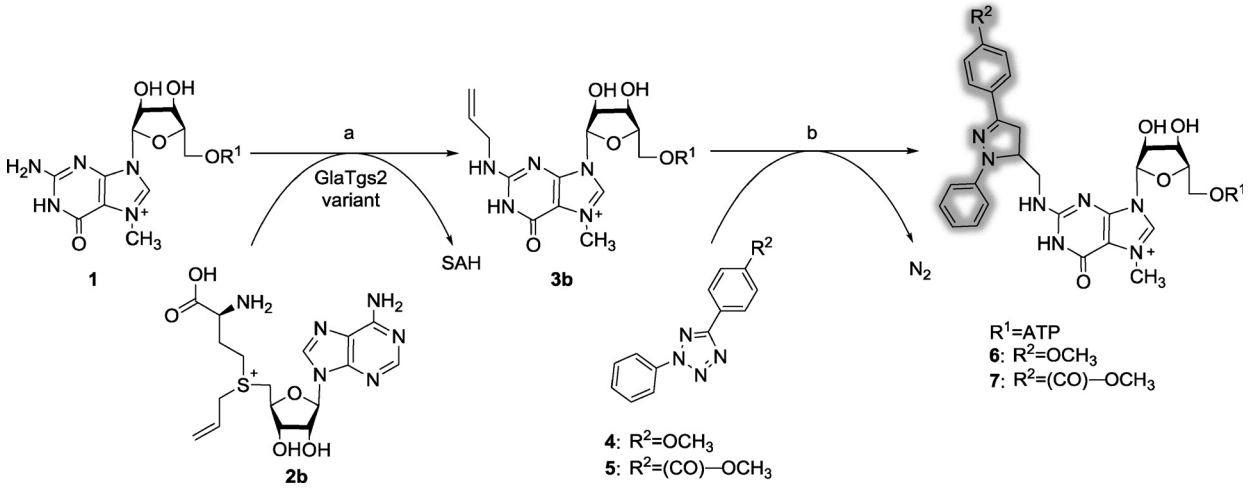
The 1,3-dipolar cycloaddition of an alkene with a nitrile imine (originally described by Huisgen and co-workers[40,41]) was recently adapted to fluorescently label proteins in living cells.[1,42] This photoclick reaction is triggered by UV light, which generates the reactive nitrile imine intermediate from a tetrazole. The photoclick reaction can be temporally and spatially controlled, is bioorthogonal,[1,2] and fluorogenic, because a fluorescent pyrazoline cycloadduct is formed from non-fluorescent reactants. Importantly, it was possible to find conditions under which the UV light required to start the reaction was not detrimental to the cells.[1,43] The photoclick reaction could provide a valuable labeling strategy for RNA. Since the allyl group can be efficiently transferred by different methyltransferases, including DNA and RNA methyltransferases, this approach will be of interest to a broader community.[30,44]

Results and Discussion

In this work we established a chemo-enzymatic approach to fluorescently label the 5′ cap typical of eukaryotic mRNAs using a photoclick reaction (Scheme 2). The 5′ cap consists of an *N*7-methylated guanosine which is linked to the next nucleotide by a 5′–5′ triphosphate bridge. It is specifically recognized by trimethylguanosine synthases, which are RNA methyltransferases that transfer one or two additional methyl groups to the exocyclic N^2.[45–47] One such enzyme, GlaTgs2, is well characterized and transfers only a single methyl group.[45,46]

The photoclick reaction developed by Lin and co-workers bears potential to provide N^2-allyl-modified cap **3b** with a fluorescent label in a bioorthogonal manner.[48] This click reaction is a type I 1,3-dipolar cycloaddition, implicating that reactivity

© 2015 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 2. Chemo-enzymatic approach for labeling the 5′ cap by the photoclick reaction. a) The capped minimal mRNA m^7GpppA (**1**) is specifically modified by a variant of GlaTgs2, which introduces the allyl residue from **2b** at position N^2, while the coproduct *S*-adenosylhomocysteine (SAH) is released. A reaction at 37 °C for 180 min yields N^2-allyl-m^7GpppA (**3b**, up to 90%). b) The product **3b** together with tetrazole **4** or **5** is irradiated at 254 nm for 5 min and left at 4 °C o/n. The 1,3-dipolar cycloaddition reaction yields fluorescent pyrazoline cycloadduct **6** or **7** (yields not determined).

is governed by the energy gap between the HOMO of the dipole (nitrile imine generated from tetrazole) and the LUMO of the dipolarophile (N^2-allylated mRNA cap).[49] Given that the symmetry of the frontier orbitals matches, the reaction of this system can be accelerated either by lowering the LUMO of the dipolarophile[50] or by lifting the HOMO energy of the nitrile imine.[49]

For a model photoclick reaction between 4-penten-1-ol and different tetrazoles, Wang et al. described how altering the HOMO energy of the nitrile imine affected the reactivity for a given alkene.[49] To demonstrate bioorthogonality of the photoclick reaction, proteins were modified with non-native amino acids bearing the required allyl group and served as substrates. These amino acids were typically *O*-allyl-modified (e.g. *O*-allyl-tyrosin) or *C*-allyl-modified (e.g. homoallylglycine, HAG). Although the wavelength for nitrile imine generation is rather short (λ_{ex} = 302 nm), the reaction was successfully performed in living cells.[1,2]

In contrast to these amino acid substrates, the 5′ cap bears a positive charge in the purine ring system due to methylation at *N*7 and 1–3 pH-dependent negative charges at the triphosphate. Furthermore, the cap bears an *N*-allyl group, whereas the substrates reported so far had *O*-allyl or *C*-allyl groups. Therefore, it was questionable whether N^2-derivatized cap structures are suitable dipolarophiles for the 1,3-dipolar cycloadditions with nitrile imines.

We chose tetrazoles **4** and **5** as nitrile imine precursors. Both **4** and **5** have been used before to fluorescently label alkene-bearing proteins—importantly, also for live-cell imaging.[1,2,49] The nitrile imines generated from these tetrazoles bear different para substituents on the *C*-aryl ring resulting in different energies of the highest occupied molecular orbitals (HOMOs) (−4.58 eV versus −5.06 eV) that were calculated by Kohn–Sham density functional theory (KS-DFT) using the BP86 functional.[49,51–57] Changing the substituent may thus provide a means to tune the reactivity of the tetrazole precursor with the dipolarophile **3**.

We used KS-DFT to calculate the energies and shapes of frontier molecular orbitals of all reactants and to estimate their reactivity. For the calculations, we used N^2-allyl-m^7GTP-H^+ (**8**) as representative model substrate for the N^2-allyl-modified cap **3b**, which functions as dipolarophile, and the nitrile imines **4a** and **5a**, respectively as dipoles (Supplementary Figure 1 in the Supporting Information).

The HOMO of the nitrile imine **4a** and the LUMO+2 of N^2-allyl-m^7GTP-H^+ **8** are shown in Figure 1. They were identified as suitable frontier orbitals because they meet the criteria for symmetry and phases required for the reaction. Although the frontier MOs (FMOs) chosen for **8** are not the overall HOMO and LUMO of the N^2-allyl-modified cap, these were selected because they are located at the N^2-allyl group involved in the reaction.

Orbitals with energies between the LUMO+2 and the HOMO−15 of **8** were predominantly located on other parts of the molecule and therefore disregarded as FMOs for the photoreaction under study. It is also plausible that the energetic ordering of the molecular orbitals and, to a lesser degree, their shapes (in particular those located on the charged part of the structure) may be affected by solvent effects, which are beyond the scope of this study.

The energies of suitable FMOs of **4a** and **8** were −4.58 eV (HOMO of **4a**) and −2.48 eV (LUMO+2 of **8**), respectively, resulting in an energy gap of 2.10 eV (Figure 1). For a reaction of the N^2-allyl-modified cap with the methoxycarbonyl-substituted tetrazole **5**, the FMOs were calculated in the same manner for the corresponding nitrile imine **5a**. An energy gap of 2.58 eV was calculated for the LUMO+2 of **8** and the HOMO of **5a** (Table 1). Our calculations suggest that tetrazoles **4** and **5** are suitable substrates for photoclick reactions with N^2-allyl-m^7GpppA (**3b**) because 1,3-dipolar cycloadditions with FMO

© 2015 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

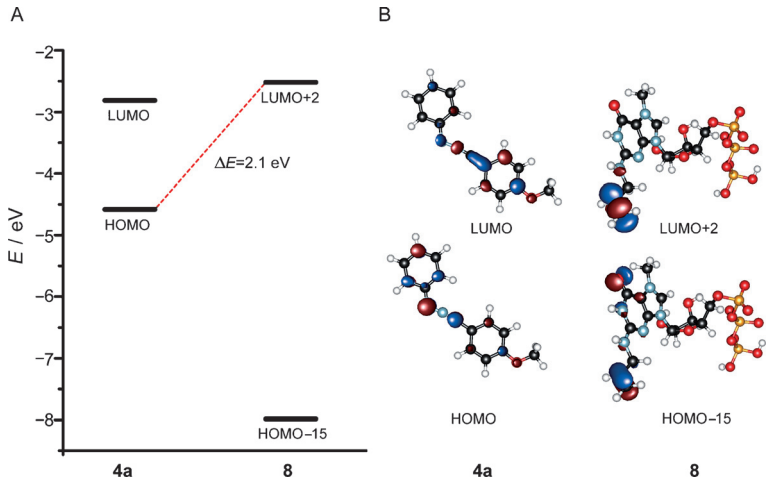


Figure 1. Energies of calculated frontier orbitals from nitrile imine **4a** and N^2-allyl-m^7GTP-H^+ (**8**) as well as isosurface plots of the corresponding molecular orbitals. A) Schematic representation of frontier orbital energies of nitrile imine **4a** as well as N^2-allyl-modified m^7GTP-H^+ (**8**) calculated by KS-DFT. Based on these calculations, the HOMO–LUMO gap of reactants is 2.1 eV. B) Orbitals with a suitable spatial structure for interaction were chosen as frontier molecular orbitals.

Table 1. Calculated energies of the frontier molecular orbitals (FMOs) of the photoclick educts.[a]

	E_{HOMO} [eV]	E_{LUMO} [eV]
4a	−4.58	−2.18
5a	−5.06	−3.05
8	−7.95 (HOMO−15)	−2.48 (LUMO+2)
acrylamide	−7.05 (HOMO−2)	−2.23

[a] The calculated energies of molecular orbitals involved in the photoclick reaction of the dipoles **4a** and **5a** as well as the dipolarophiles **8** and acrylamide are summarized.

gaps of up to 10 eV have been reported in the literature.[58,59] The shortcomings of a single-particle (MO) picture, solvent effects and the choice of the approximate exchange-correlation functional will have a significant effect, so our calculations should be interpreted as giving a qualitative picture. To illustrate the dependence on the functional, we also carried out calculations with the hybrid functional B3LYP (see Supplementary Table 1 in the Supporting Information). While the absolute orbital energies do change as expected (showing broader HOMO–LUMO gaps for B3LYP compared with BP86), our qualitative conclusions remain unaffected (energy gap to LUMO+2 of **8** is 3.49 eV for **4a** versus 3.93 eV for **5a**). Overall, the calculations indicate that the methoxy-substituted tetrazole **4** is a better substrate than the methoxycarbonyl-substituted tetrazole **5**, because the HOMO of the resulting nitrile imine **4a** is higher in energy and thus the energy gap of the FMOs considering **8** as dipolarophile is lower.

Since experiments with the N^2-allyl-modified cap are costly, we sought to establish a model reaction first. Therefore, we also calculated the energy gap for the reaction of our nitrile imines with acrylamide **9**. The resulting energy gaps were 2.35 eV and 2.83 eV for reaction with **4a** and **5a** (Table 1), respectively, and thus in the same range as for the N^2-allyl-modified cap **8**, indicating that acrylamide can be used as a simple model substrate to establish the photoclick reaction.

We synthesized both **4** and **5** as described by Ito et al.[60] and characterized them using electrospray ionization mass spectrometry (ESI-MS) and ^{1}H NMR (data in the Supporting Information). Both tetrazoles were then used in photoclick reactions with the model substrate acrylamide as described by Song et al.[2] The fluorescence spectra of the resulting products **10** and **11** showed emission maxima of 460 nm and 525 nm, respectively, whereas control reactions (without acrylamide) showed no fluorescence (Supplementary Figure 2 in the Supporting Information). Thus, different substituents at the tetrazole ring changed the fluorescence spectra of the resulting pyrazoline products. These data are in accordance with the results described by Lin and co-workers.[61] Since the LUMO energy of **8** is even lower compared with acrylamide (Table 1), our model reactions suggest that photoclick chemistry with tetrazoles **4** and **5** can be used to label the N^2-allyl cap.

We next sought to apply the reaction to the allyl-modified cap **3b** to establish a new strategy for bioorthogonal labeling of the 5′ cap. For our two-step approach, we first produced N^2-allyl-m^7GpppA (**3b**) from m^7GpppA (**1**) by enzymatic transfer of an allyl group using the cosubstrate AdoPropen (**2b**). According to our KS-DFT calculations, tetrazoles **4** and **5** should be suitable for this purpose. We used a previously engineered variant, GlaTgs2-V34A to obtain up to 90% (900 μM) N^2-allyl-m^7GpppA (**3b**) at a catalyst loading of up to 0.7 mol%. After heating the samples and dialysis, the resulting N^2-allyl-m^7GpppA (**3b**) was used as substrate in a photoclick reaction with tetrazole **4**.

After irradiation at 254 nm and incubation at 4 °C overnight, the reaction mixture was separated by denaturing 20% polyacrylamide gel electrophoresis (PAGE), and in-gel fluorescence was detected by a Casio Exilim 12.5x camera for 8 s upon excitation at 365 nm using a hand-held UV lamp (Figure 2). Control reactions in the absence of cap, enzyme, AdoPropen (**2b**), or active enzyme were performed.

We observed a new, cyan-fluorescent band exclusively in the reaction yielding N^2-allyl-modified cap **3b** (lane 1, Figure 2B). Control reactions lacking one of the required components did not give this band (lanes 2–5 in Figure 2B). These controls were performed without (active) enzyme, without m^7GpppA (**1**), or without cosubstrate **2b**, respectively, and could not yield **3b**. The new band in lane 1 could be assigned to the expected pyrazoline product **6** using high-performance liquid chromatography coupled with electrospray ionization time-of-

© 2015 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

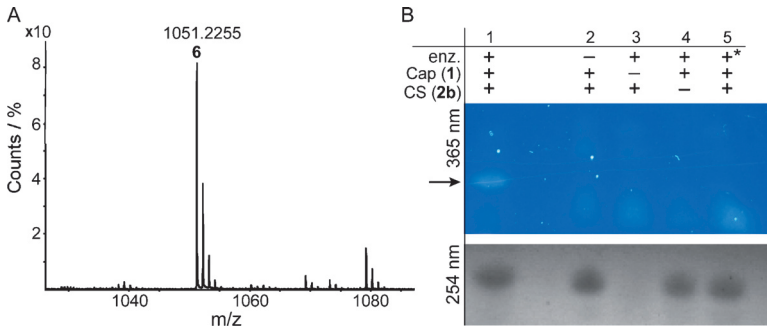


Figure 2. Analysis of the photoclick reaction of tetrazole **4** with N^2-allyl-m^{7}GpppA (**3b**). The cap analog m^{7}GpppA (**1**) was converted with the GlaTgs2 variant and cosubstrate (CS) **2b** to yield **3b**, which was subsequently used for photoclick reaction with **4**. A) ESI-MS analysis of the photoclick reaction of **3b** with **4**. The mass of the expected product **6** was detected ($[M]^+ = 1051.23$). B) Reaction mixtures were separated by PAGE and analyzed for the formation of a fluorescent pyrazoline cycloadduct. Upper panel: fluorescent signals upon irradiation at 365 nm. Lane 1 contains an additional fluorescent band (arrow). All lanes show a blue fluorescent band at the bottom (side product from tetrazole). Lower panel: UV shadowing of the same gel as loading control showing N^2-allyl-m^{7}GpppA (**3b**) in lane 1 or m^{7}GpppA (**1**) in lanes 2, 4, and 5. The photoclick product **6** was not detectable by UV shadowing. The sample in lane 5 used a denatured enzyme (+*).

flight mass spectrometry (HPLC-ESI-TOF-MS) (Figure 2A and Supplementary Figure 3 in the Supporting Information). However, the reaction and the controls contained an additional fluorescent band with higher electromobility. This band was also observed after irradiation of tetrazole alone (lane 5 of Supplementary Figure 4 in the Supporting Information), and therefore likely originates from side products formed from tetrazole after irradiation.

Next, we wanted to test whether the differently substituted tetrazole **5** can also react with **3b** in a photoclick reaction. Based on our calculations above, the photoclick reaction should also proceed with tetrazole **5**, albeit slower, because the HOMO (dipole)–LUMO (dipolarophile) gap is higher compared with the reaction of **4** with **3b**. Our experiments confirmed that **3b** and **5** can react in a photoclick reaction as we observed a fluorescent product in the reaction, which was absent in all controls (Supplementary Figure 5 in the Supporting Information). Interestingly, higher tetrazole concentrations were required to make the reaction work (1.23 mM instead of 0.62 mM), indicating that the reactivity of the N^2-allyl-modified cap **3b** with **5** is lower—as expected based on our calculations for **8**.

We thus demonstrated that the 5′ cap can be labeled with a turn-on fluorescent probe by installing an N^2-allyl moiety and a subsequent bioorthogonal photoclick reaction. KS-DFT allowed us to calculate the FMO energies and was suitable to predict the reactivities of the participating molecules.

We could show above that increasing the HOMO energy of the nitrile imine by altering the *para*-substituent of the phenyl ring improved the reaction of the respective tetrazole precursors with the N^2-allyl-modified cap **3b**. This was expected because increasing the HOMO energy of the dipole decreases the HOMO (dipole)–LUMO (dipolarophile) gap of the frontier orbitals.

The alternative way to decrease this energy gap would be to lower the energy of the LUMO of the dipolarophile, that is, the N^2-substituted cap. As a conjugated multiple bond is known to exhibit higher HOMO and lower LUMO energy compared with an unconjugated one,[62] we hypothesized that appending an alkyne to the alkene moiety of the dipolarophile should lower its LUMO, thereby enhancing its reactivity in a photoclick reaction.

Calculating the FMO-energies of N^2-pentenynyl-m^{7}GTP-H$^+$ (**12**, structure in Supplementary Figure 1 in the Supporting Information) we found a LUMO+1 energy of only −3.26 eV, concomitant with an energy gap of only 1.32 eV between the HOMO of **4a** and the LUMO of **12**. These calculations suggest that a pentenynyl-substituted cap may be a better substrate for the photoclick reaction with **4**. We therefore produced N^2-pentenynyl-modified m^{7}GpppA **3c** by enzymatic modification of m^{7}GpppA (**1**) using the GlaTgs2 variant and the cosubstrate 5′-[(*R*/*S*)(3*S*)-3-amino-3-carboxypropyl]pent-2-en-4-ynylsulfonio]-5′-deoxyadenosine (AdoEnYn, **2c**), as described previously (~25% yield).[30] As expected, **3c** reacted with tetrazole **4** in a photoclick reaction to form a fluorescent product that could be detected by in-gel fluorescence, although the band is rather faint (Figure 3). We presume the low concentration of modified cap **3c** in bioconversions (about 200 μM) to be the limiting factor in this case for the photoclick reaction. A control reaction was carried out under identical conditions, but with denatured enzyme, and did not give a new fluorescent product (Figure 3). These data, together with our calculations, suggest that the expected product **13** was formed, confirming that KS-DFT calculations are useful to predict reactivity between a nitrile imine and a modified cap acting as a dipolarophile.

Conclusions

We developed a novel chemo-enzymatic approach for labeling eukaryotic mRNA 5′ caps using photoclick chemistry with a potential application in living cells. To achieve this, we first predicted reactivities of two nitrile imines with the model substrate N^2-allyl-m^{7}GTP-H$^+$ (**8**) by Kohn–Sham DFT (KS-DFT) calculations of frontier molecular orbital (FMO) energies. The energy gaps between suitable FMOs were determined to be 2.10 eV (for **4a**) and 2.58 eV (for **5a**), respectively when using the BP86 functional, and 3.49 eV (for **4a**) and 3.93 eV (for **5a**), respectively, when employing B3LYP. These calculations revealed that cycloadditions of the N^2-allylated mRNA cap with both tetrazoles should be possible, but kinetically favored with **4**.

To demonstrate the feasibility of mRNA fluorescent labeling by a photoclick reaction, we labeled the minimal mRNA m^{7}GpppA (**1**) by enzymatic alkenylation and subsequent photoinduced 1,3-dipolar cycloaddition with tetrazole **4** in vitro. In

© 2015 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

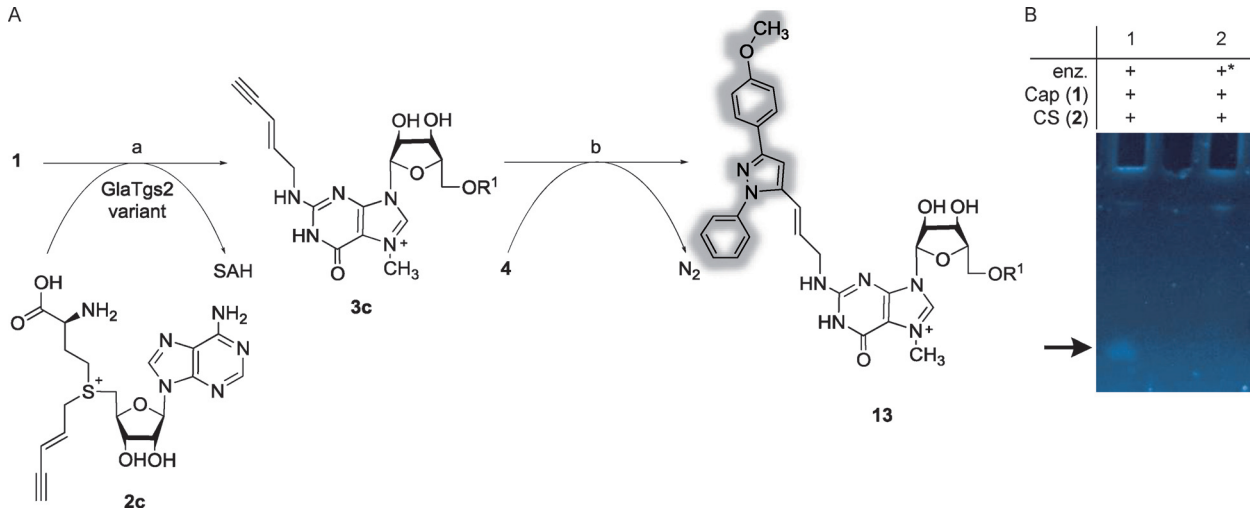


Figure 3. Analysis of the photoclick reaction of tetrazole **4** with N^2-pentenynyl-m^{7}GpppA (**3c**). A) a) The cap-analog m^{7}GpppA (**1**) was used with the GlaTgs2 variant and cosubstrate (CS) **2c** to give N^2-pentenynyl-m^{7}GpppA (**3c**), which was subsequently used for photoclick reaction with **4**. b) The product **3c** together with tetrazole **4** is irradiated at 254 nm for 5 min and left at 4 °C o/n. to give **13** (yield not determined). B) Reaction mixtures were separated by PAGE and analyzed for the formation of a fluorescent pyrazoline cycloadduct, visible upon irradiation at 365 nm. A fluorescent band is observed in lane 1 (arrow), which is absent in the control reaction containing denatured enzyme (+*, lane 2).

this experiment, a new fluorescent product was observed after separation by PAGE. The formation of the expected pyrazoline **6**, was confirmed by mass spectrometry. Cycloaddition was also successfully performed with **5**, but with lower reactivity, as suggested by KS-DFT calculations.

Furthermore, we experimentally validated that fluorescent labeling of an mRNA cap bearing a conjugated triple bond at N^2 is feasible, as predicted by KS-DFT calculations. The yields obtained by allyl- versus pentenynyl-modified 5′ caps should not be compared with the calculated differences in energy because multiple factors contribute (e.g. different yields in the enzymatic reactions with the two substrates), and kinetic and thermodynamic effects should be regarded separately.

In summary, we demonstrated that fluorescent labeling of the 5′ cap—a hallmark of eukaryotic mRNAs—can be achieved by enzymatic alkenylation (or alkynylation) followed by the bioorthogonal photoclick reaction. Furthermore, our results emphasize that reactivity of nitrile imines with complex molecules like the N^2-modified mRNA 5′ cap can be correctly predicted by in silico studies. This chemo-enzymatic approach unites several desirable features for mRNA labeling, like the possibility 1) to generate fluorescent products from non-fluorescent educts, 2) to use light as trigger, as well as 3) to fluorescently label endogenous mRNAs by introducing small groups, thus minimizing perturbation of the native system.

Importantly, photoclick chemistry between tetrazoles and terminal alkenes is described as bioorthogonal and has already been successfully applied for labeling of proteins in living cells.[1,2] The reported approach thus presents a step forward regarding applicability in living cells compared with previously reported labeling approaches for the mRNA cap relying on CuAAC, SPAAC, or thiol-ene click reactions.[30,31] However, we have only used the minimal cap in the photoclick reaction. Nevertheless, we anticipate that the approach will also work for longer capped RNA, for example eukaryotic mRNAs. Depending on the residues attached to the tetrazole reagent, our approach may then be used for isolation, but also for visualization of mRNAs in the future. For the long-term perspective of live-cell experiments, numerous improvements will be required. The yield of the photoclick reaction and the detection limit will have to be greatly improved to make this reaction applicable to RNA labeling in cells. A human cell is typically 100–10,000 μm^3 big and contains, on average, approximately 300,000 mRNA molecules.[63] Although mRNAs are not homogenously distributed in a eukaryotic cell but are enriched in foci and bodies, and calculated concentrations in a compartmented cells should be treated with care, it is clear that the currently used concentrations of 5′ cap will have to be significantly decreased (200 μM m^{7}GpppA (**1**) yielding 180 μM of the N^2-allyl-m^{7}GpppA (**3b**), which is the dipolarophile for the photoclick reaction).[45]

Nonetheless, the successful fluorescent labeling of the eukaryotic mRNA 5′ cap by the bioorthogonal photoclick reaction and the possibility to predict reactivity between cap and nitrile imine in silico provide a promising starting point to establish novel strategies for the isolation of eukaryotic mRNAs and their visualization in living cells.

Experimental Section

Experimental details can be found in the Supporting Information.

Acknowledgements

The authors acknowledge the assistance of the mass spectrometry facility of the University of Hamburg and would like to thank Prof. Zhaohui "Sunny" Zhou (Northeastern University, Boston, MA, USA) as well as Prof. Birgit Dräger (University of Halle, Germany) for plasmids encoding LuxS and MTAN. A. R. gratefully acknowl-

 © 2015 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

edges financial support from the Emmy Noether-Programme of the Deutsche Forschungsgemeinschaft (DFG) (RE 2796/2–1). C. H. and A. R. thank the Fonds der Chemischen Industrie for financial support. This work was partly supported by the DFG EXC 1003 Cells in Motion–Cluster of Excellence, Münster, Germany.

Keywords: bioorthogonal labeling · fluorescent labeling · Kohn–Sham DFT calculations · 5′ cap · mRNA · photoclick reactions

[1] W. Song, Y. Wang, Z. Yu, C. I. R. Vera, J. Qu, Q. Lin, *ACS Chem. Biol.* **2010**, *5*, 875 – 885.
[2] W. Song, Y. Wang, J. Qu, Q. Lin, *J. Am. Chem. Soc.* **2008**, *130*, 9654 – 9655.
[3] A. Niederwieser, A.-K. Späte, L. D. Nguyen, C. Jüngst, W. Reutter, V. Wittmann, *Angew. Chem. Int. Ed.* **2013**, *52*, 4265 – 4268; *Angew. Chem.* **2013**, *125*, 4359 – 4363.
[4] N. Li, K. V. Lim, S. Edwardraja, Q. Lin, *J. Am. Chem. Soc.* **2011**, *133*, 15316 – 15319.
[5] D. Schulz, A. Rentmeister in *Chem. Biol. Nucleic Acids* (Eds.: V. A. Erdmann, W. T. Markiewicz, J. Barciszewski), Springer, Berlin, Heidelberg, **2014**, pp. 409 – 422.
[6] K. C. Martin, A. Ephrussi, *Cell* **2009**, *136*, 719 – 730.
[7] J. C. Politz, E. S. Browne, D. E. Wolf, T. Pederson, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6043 – 6048.
[8] C. Molenaar, A. Abdulle, A. Gena, H. J. Tanke, R. W. Dirks, *J. Cell Biol.* **2004**, *165*, 191 – 202.
[9] T. Matsuo, *Biochim. Biophys. Acta Gen. Subj.* **1998**, *1379*, 178 – 184.
[10] J. S. Rinne, T. P. Kaminski, U. Kubitscheck, A. Heckel, *Chem. Commun.* **2013**, *49*, 5375 – 4377.
[11] E. Bertrand, P. Chartrand, M. Schaefer, S. M. Shenoy, R. H. Singer, R. M. Long, *Mol. Cell* **1998**, *2*, 437 – 445.
[12] N. Daigle, J. Ellenberg, *Nat. Methods* **2007**, *4*, 633 – 636.
[13] K. M. Forrest, E. R. Gavis, *Curr. Biol.* **2003**, *13*, 1159 – 1168.
[14] D. Fusco, N. Accornero, B. Lavoie, S. M. Shenoy, J. M. Blanchard, R. H. Singer, E. Bertrand, *Curr. Biol.* **2003**, *13*, 161 – 167.
[15] A. K. Rath, A. Rentmeister, *Curr. Opin. Biotechnol.* **2015**, *31*, 42 – 49.
[16] J. S. Paige, K. Wu, S. R. Jaffrey, *Science* **2011**, *333*, 642 – 646.
[17] R. L. Strack, M. D. Disney, S. R. Jaffrey, *Nat. Methods* **2013**, *10*, 1219 – 1224.
[18] S. J. Kellermann, A. K. Rath, A. Rentmeister, *ChemBioChem* **2013**, *14*, 200 – 204.
[19] T. Ozawa, Y. Natori, M. Sato, Y. Umezawa, *Nat. Methods* **2007**, *4*, 413 – 419.
[20] H. Yoshimura, A. Inaguma, T. Yamada, T. Ozawa, *ACS Chem. Biol.* **2012**, *7*, 999 – 1005.
[21] A. K. Rath, S. J. Kellermann, A. Rentmeister, *Chem. Asian J.* **2014**, *9*, 2045 – 2051.
[22] S. Cabantous, H. B. Nguyen, J.-D. Pedelacq, F. Koraïchi, A. Chaudhary, K. Ganguly, M. A. Lockard, G. Favre, T. C. Terwilliger, G. S. Waldo, *Sci. Rep.* **2013**, *3*, 2854 – 2862.
[23] J. Tilsner, O. Linnik, M. Louveaux, I. M. Roberts, S. N. Chapman, K. J. Oparka, *J. Cell Biol.* **2013**, *201*, 981 – 995.
[24] M. Tomkuviene, B. Clouet-d'Orval, I. Cerniauskas, E. Weinhold, S. Klimasauskas, *Nucleic Acids Res.* **2012**, *40*, 6765 – 6773.
[25] Y. Motorin, J. Burhenne, R. Teimer, K. Koynov, S. Willnow, E. Weinhold, M. Helm, *Nucleic Acids Res.* **2011**, *39*, 1943 – 1952.
[26] W. Peters, S. Willnow, M. Duisken, H. Kleine, T. Macherey, K. E. Duncan, D. W. Litchfield, B. Lüscher, E. Weinhold, *Angew. Chem. Int. Ed.* **2010**, *49*, 5170 – 5173; *Angew. Chem.* **2010**, *122*, 5296 – 5299.
[27] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046 – 15047.
[28] G. Lukinavičius, V. Lapiené, Z. Stasevskij, C. Dalhoff, E. Weinhold, S. Klimašauskas, *J. Am. Chem. Soc.* **2007**, *129*, 2758 – 2759.
[29] C. Y. Jao, A. Salic, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 15779 – 15784.
[30] D. Schulz, J. M. Holstein, A. Rentmeister, *Angew. Chem. Int. Ed.* **2013**, *52*, 7874 – 7878; *Angew. Chem.* **2013**, *125*, 8028 – 8032.
[31] J. M. Holstein, D. Schulz, A. Rentmeister, *Chem. Commun.* **2014**, *50*, 4478 – 4481.
[32] D. Schulz, A. Rentmeister, *ChemBioChem* **2014**, *15*, 2342 – 1347.
[33] A. M. Pyka, C. Domnick, F. Braun, S. Kath-Schorr, *Bioconjugate Chem. Bioconjug. Chem.* **2014**, *25*, 1438 – 1443.
[34] J. Schoch, S. Ameta, A. Jäschke, *Chem. Commun.* **2011**, *47*, 12536 – 12537.
[35] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599; *Angew. Chem.* **2002**, *114*, 2708 – 2711.
[36] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057 – 3064.
[37] M.-L. Winz, A. Samanta, D. Benzinger, A. Jäschke, *Nucleic Acids Res.* **2012**, *40*, e78.
[38] D. C. Kennedy, C. S. McKay, M. C. B. Legault, D. C. Danielson, J. A. Blake, A. F. Pegoraro, A. Stolow, Z. Mester, J. P. Pezacki, *J. Am. Chem. Soc.* **2011**, *133*, 17993 – 8001.
[39] E. J. Kim, D. W. Kang, H. F. Leucke, M. R. Bond, S. Ghosh, D. C. Love, J.-S. Ahn, D.-O. Kang, J. A. Hanover, *Carbohydr. Res.* **2013**, *377*, 18 – 27.
[40] R. Huisgen, M. Seidel, G. N. Wallbillich, *Tetrahedron* **1962**, *17*, 3 – 29.
[41] J. S. Clovis, A. Eckell, R. Huisgen, R. Sustmann, *Chem. Ber.* **1967**, *100*, 60 – 70.
[42] Y. Wang, C. I. R. Vera, Q. Lin, *Org. Lett.* **2007**, *9*, 4155 – 4158.
[43] Z. Yu, L. Y. Ho, Z. Wang, Q. Lin, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5033 – 5036.
[44] C. Dalhoff, G. Lukinavicius, S. Klimasăuskas, E. Weinhold, *Nat. Chem. Biol.* **2006**, *2*, 31 – 32.
[45] S. Hausmann, S. Shuman, *J. Biol. Chem.* **2005**, *280*, 32101 – 32106.
[46] D. Benarroch, M. Jankowska-Anyszka, J. Stepinski, E. Darzynkiewicz, S. Shuman, *RNA* **2010**, *16*, 211 – 220.
[47] T. Monecke, A. Dickmanns, A. Strasser, R. Ficner, *Acta Crystallogr. Sect. D* **2009**, *65*, 332 – 338.
[48] W. Song, Y. Wang, J. Qu, M. M. Madden, Q. Lin, *Angew. Chem. Int. Ed.* **2008**, *47*, 2832 – 2835; *Angew. Chem.* **2008**, *120*, 2874 – 2877.
[49] Y. Wang, W. Song, W. J. Hu, Q. Lin, *Angew. Chem. Int. Ed.* **2009**, *48*, 5330 – 5333; *Angew. Chem.* **2009**, *121*, 5434 – 5437.
[50] K. N. Houk, J. Sims, C. R. Watts, L. J. Luskus, *J. Am. Chem. Soc.* **1973**, *95*, 7301 – 7315.
[51] R. Ahlrichs, M. Bar, H. Marco, H. Horn, C. Ktjlmel, *Chem. Phys. Lett.* **1989**, *162*, 165 – 169.
[52] http://www.turbomole.com, **2009**.
[53] S. Wittrock, T. Becker, H. Kunz, *Angew. Chem. Int. Ed.* **2007**, *46*, 5226 – 5230; *Angew. Chem.* **2007**, *119*, 5319 – 5323.
[54] A. D. Becke, *Phys. Rev. A* **1988**, *38*, 3098 – 3100.
[55] J. P. Perdew, *Phys. Rev. B* **1986**, *33*, 8822 – 8824.
[56] ftp://ftp.chemie.uni-karlsruhe.de/pub/jbasen, **2006**.
[57] A. Schäfer, C. Huber, R. Ahlrichs, *J. Chem. Phys.* **1994**, *100*, 5829.
[58] A. Ponti, G. Molteni, *New J. Chem.* **2002**, *26*, 1346 – 1351.
[59] G. Molteni, A. Ponti, M. Orlandi, *New J. Chem.* **2002**, *26*, 1340 – 1345.
[60] S. Ito, Y. Tanaka, A. Kakehi, K. Kondo, *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1920 – 1923.
[61] Z. Yu, L. Y. Ho, Q. Lin, *J. Am. Chem. Soc.* **2011**, *133*, 11912 – 11915.
[62] R. Sustmann, *Pure Appl. Chem.* **1974**, *40*, 569 – 593.
[63] V. E. Velculescu, S. L. Madden, L. Zhang, A. E. Lash, Y. Jian, C. Rago, A. Lal, C. J. Wang, G. A. Beaudry, K. M. Ciriello, B. P. Cook, M. R. Dufault, A. T. Ferguson, Y. Gao, T. C. He, H. Hermeking, S. K. Hiraldo, P. M. Hwang, M. A. Lopez, H. F. Luderer, B. Mathews, J. M. Petroziello, K. Polyak, L. Zawel, K. W. Kinzler, *Nat. Genet.* **1999**, *23*, 387 – 388.

Received: November 7, 2014
Published online on February 4, 2015

 © 2015 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim