Click Chemistry

DOI: 10.1002/anie.200604897

The Click Reaction in the Luminescent Probing of Metal Ions, and Its Implications on Biolabeling Techniques

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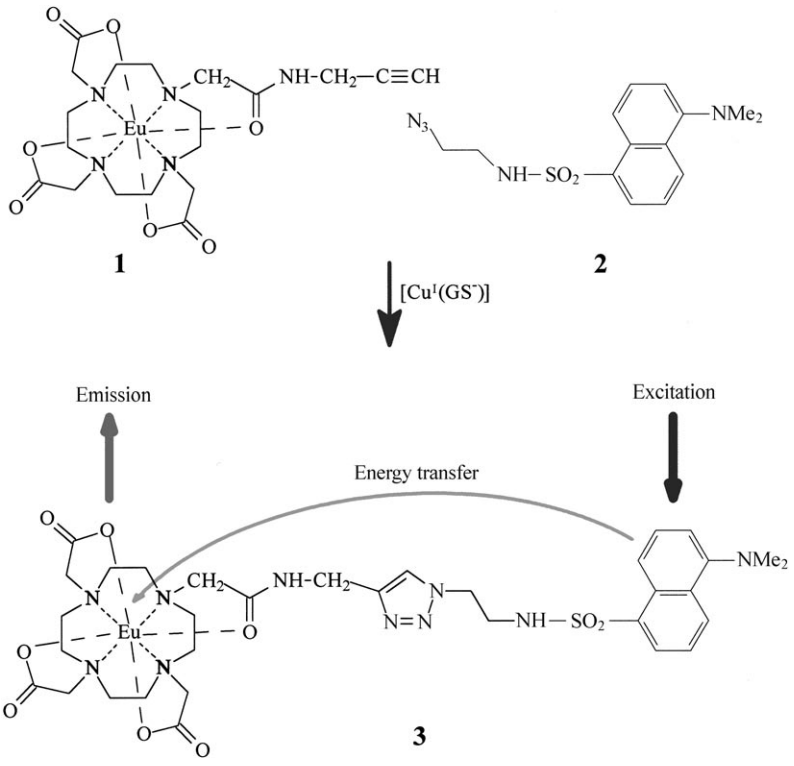
Keywords:
analytical methods · cell assays · click chemistry · copper · fluorescent probes

A common way to probe metal ions by optical means is based on the use of metallochromic molecular indicators (more commonly referred to as probes or molecular sensors). On binding to ions, these undergo a detectable change in color, in fluorescence intensity, or fluorescence lifetime. Optical probes enable the detection (qualitative) and determination (quantitative) of ions in samples where electroanalytical methods are not easily applicable, for example, in cells and tissue, and in particular, in terms of spatially resolved probing (i.e. imaging). Given the role that such ions play in biochemistry, there is substantial activity in this field in developing new methods. Recently, methods have been introduced that are based on the catalytic effect that certain ions (or molecules) exert on classical chemical reactions.

It was reported[1] that copper(I) ions, in even micromolar concentrations, catalyze the 1,3-dipolar cycloaddition of alkynyl groups to azido groups to form a triazole (see Scheme 1), a reaction discovered by Huisgen some decades ago. Specifically, the alkynyl group of the Eu^{3+} tetraazacyclododecane complex **1** was reacted with the azido group of the fluorophore **2** to give the conjugate **3**, which emits red luminescence as a result of ligand-to-metal energy transfer (LMET). The catalytic effect of Cu^{+} on this cycloaddition was independently discovered by the groups of Meldal[2] and Sharpless,[3] and it is often referred to as the "click" reaction. Notably, the reagents used are available in a reasonable number of synthetic steps.

The formation of **3** was monitored over 1 h through the increase in the luminescence intensity of the Eu^{3+} ion peak at 616 nm. The signal change is attributed to the fact that following cycloaddition the 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) fluorophore, which acts as a light-harvesting (antenna group), and the central Eu^{3+} metal ion lie within a distance over which LMET can occur. The reaction occurs under physiological conditions (i.e. in aqueous solution at near-neutral pH and at room temperature)[4] and therefore can be used to detect the presence of Cu^{+} ions in cells. This ion (along with its bivalent analogue) is required as a cofactor in almost 20 enzymatically catalyzed reactions.

Furthermore, the reaction leads to an increase (rather than a decrease) in luminescence intensity. Interestingly,



Scheme 1. The rate of the cycloaddition of alkyne **1** to the azide **2** is determined by the concentration of the catalytically active Cu^{+}-gluthathione ($Cu^{I}GS^{-}$) complex. The product **3** has a characteristic red luminescence that is not observed in the starting reagents at an excitation wavelength of 350 nm.

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quenching (rather than an increase) of the luminescence was observed in a rather similar compound,[5] a fact that underpins the dramatic effect that the distance between two fluorophores can have on the photophysical and luminescent properties of such a system.

The reaction shown in Scheme 1 is catalyzed not only by free Cu^+ but also by glutathione-bound (rather than free) Cu^+ and is in striking contrast to most conventional methods[6] that are based on ion chelators and sometimes can detect solvent-exposed ions only but not buried or bound ones. This feature is of great significance in terms of the concentration of active ions inside cells. In fact, the effective concentration of free copper ions in the cytoplasm is under a single ion per cell, but the concentration of bound copper ions is on the order of micromoles per liter!

The method described above represents a quite new approach towards sensing Cu^+ ions; it is likely to work for any ion capable of catalyzing organic chemical reactions, and it is principally different from probes that rely on the chelation of ions. Rather, it is related to an approach reported earlier by Anslyn and Zhu,[7] who developed a method in which a catalytic reaction is used for signal amplification through the induction of fluorescence resonance energy transfer (FRET). The general philosophy in this case is that any regulatory element (such as a catalyst or an inhibitor) of a given chemical reaction can form the basis for converting it into an analytical method. If the two partners of the reaction (whose rate is governed by the regulatory element) are fluorescently labeled, the reaction may lead to a product that comprises a FRET donor and a FRET acceptor and which, in turn, may lead to measurable FRET. Indeed, this was demonstrated for the case of a Cu^+-catalyzed click reaction.[7]

One attractive feature of both the LMET and the FRET methods is that they are self-referenced, which is highly desirable in life sciences as such methods are much more easily calibrated. Moreover, the Eu-based LMET approach is likely to work in the lifetime (and time-resolved) domain, while the FRET system is likely to work in the two-wavelength ratiometric mode.

Although the click method was demonstrated for the specific case of detecting ("sensing")[8] Cu^+ ions, the method has a much larger potential in terms of probing metal ions. Conceivably, it may be extended to the following:

a) alkynylated fluorophores with various colors, decay times, polarizability, and solvatochromism that may be coupled to a donor or an acceptor fluorophore that bears an azido group; this would enable an adjustment of absorption and emission wavelengths, decay times, and degrees of polarization;
b) to other catalytically active ions of interest in the biosciences, provided that the catalytic reactions occur under physiological conditions;
c) to organic catalysts (preferably, of course, if catalytically active in low concentrations), such as aniline,[9] ureas,[10] and others.[11]

If such reactions occur under physiological conditions, they will pave the way towards probing metal ions in vivo, provided that the probes are cell-permeable. It should be kept in mind, though, that such approaches towards ion sensing are irreversible, in contrast to the reversibility of many indicator-based approaches. Note also that many of the "old" spot tests that were described by Feigl[12] and were often of the catalytic type may experience a revival and an extension of their applicability.

The click method also has a substantial potential in terms of bioconjugation and surface modification. Click-type reactions can be used, for example, to label biomolecules fluorescently (or otherwise). As azides and alkynes are essentially absent from most cells, the click ligation can be quite selective. There has been some promising work in this direction already: Wang et al.[13] have conjugated a label (that carries an alkynyl group) to a protein that carries an azido group (Figure 1). Covalent bond formation was brought about by the addition of catalytic quantities of Cu^+ ion. It was noted, however, that the chelating ligand that was used to stabilize the Cu^+ oxidation state played a crucial role. On the basis of this approach, it is conceivable that numerous related labeling methods will become possible.[14]

Bertozzi and co-workers[15] have also demonstrated that cell surfaces can be genetically engineered, so that they contain saccharide units bearing azido groups. Link and Turell,[16] in turn, have demonstrated that cell surfaces can be labeled by a click reaction if the recombinant outer cell membrane of *E. coli* is expressed in the presence of the non-natural amino acid azidohomoalanine, which acts as a surrogate for methionine. The surface-exposed azido units were then conjugated to biotin through the click reaction, and the biotin unit was labeled with fluorescently tagged streptavidin.

In an extension of the click approach, Zhou and Fahrni[17] have exploited the electron-donating properties of the triazole ring formed in the click reaction to modulate the fluorescence of a coumarin fluorophore; it was found to undergo a large increase in intensity upon ligation to the azide. Tirrell and co-workers[18] have used click chemistry to fluorescently visualize a synthetic protein by labeling it with an azidocoumarin. Carell and co-workers[19] demonstrated that alkyne-modified DNA oligomers can be postsynthetically labeled with an easily accessible azido-modified fluorescein using the click reaction. The potential of the click reaction in terms of surface modification and its applications to material science has been reviewed very recently.[20] Microcontact printing and patterning of solid surfaces of arrays is another promising field of application.[21]

These reports provide novel and highly perspective approaches for labeling (and thus visualizing) biomolecules,

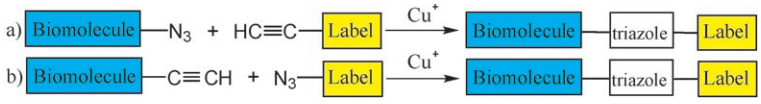

Figure 1. Schematic of bioconjugation chemistries based on the click reaction. Spacer groups between the biomolecule and alkynyl/azido groups are likely to be useful. The type of label is not limited to those that are fluorescent.

cell surfaces, and even particles, as was shown recently for the case of gold nanoparticles whose surfaces were click-modified with enzymes[22] and with chemical functionalities.[23] Signal amplification and transduction may even be achieved by photocatalyzed fluorogenic processes that, in turn, trigger a click reaction.[24]

Fluorescent labeling is but one (although quite important) way of labeling; radioactive markers, NMR contrast agents (paving the way to new approaches in MRI), isotopes (to enable bioassays based on mass spectroscopy), or enzymatic labeling (as used in enzyme-linked immunoassays, ELISA) may, of course, also be employed.

Future work in terms of the fluorescent detection of intracellular ions will have to demonstrate that:

1) adequate specificity is provided for the ion of interest,
2) the brightness of a probe (*Bs*; defined as the product of molar absorbance of the fluorophore at the wavelength of excitation and its quantum yield) is adequate for it to be of practical use in the biosciences (the *Bs* value ideally is larger than $30\,000\,\mathrm{M^{-1}\,cm^{-1}}$),
3) the probes are cell-permeable,
4) excitation wavelengths lie where the intrinsic (background) luminescence of biological systems is not as strong as under excitation in the near-UV or—even worse—the far-UV range.

Published online: March 6, 2007

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[4] Regrettably, numerous probes have been reported in the more recent literature that seem to work in organic solvents only. This, unfortunately, is of little significance to biology unless functionality also is demonstrated for aqueous solutions at near-neutral pH and at physiological temperatures.

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