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COMMUNICATION

Site specific chemoselective labelling of proteins with robust and highly sensitive Ru(II) bathophenanthroline complexes†

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The bioorthogonal and chemoselective fluorescence labelling of several cell-free synthesized proteins containing a site-specifically incorporated azido amino acid was possible using different alkyne-functionalized Ru(II) bathophenanthroline complexes. We were able to achieve a selective labelling even in complex mixtures of proteins despite the fact that ruthenium dyes normally show a high tendency for unspecific interactions with proteins and are commonly used for total staining of proteins. Since the employed Ru complexes are extremely robust, photo-stable and highly sensitive, the approach should be applicable to the production of labelled proteins for single molecule spectroscopy and fluorescence-based interaction studies.

Introduction

Site-specific labelling of proteins with chemical and fluorescent probes is a prerequisite for many established and emerging techniques in molecular and cell biology.[1] It can only be achieved by the introduction of non-naturally occurring functional groups allowing for bioorthogonal reactions.[2] Among such possibilities, the insertion of azide groups has gained increasing importance since it is prone to a chemoselective [3 + 2]-cycloaddition with an alkyne leading to triazoles, which was dubbed as the click reaction.[3,4] Alternatively, azides can also be used for a chemoselective Staudinger phosphite reaction leading to phosphoramidates.[5–7] Both processes are compatible with proteins.

There are several approaches possible to obtain proteins equipped with an azido functionality. The most common one is the suppressor approach, in which the translation yielding the protein is performed in the presence of a suitable tRNA carrying an azido-modified amino acid.[8–12]

Ru(II) bathophenanthroline complexes are chemically and thermodynamically stable and are not prone to the loss of ligands at low concentrations. After excitation by light pulses of short duration at about 440 nm they reveal a strong and long-lasting luminescence with a maximum at about 620 nm due to a metal to ligand charge transfer (MLCT) and the spin-forbidden return to the ground state. This allows for their detection at very low concentrations by time-resolved measurement techniques.[13] In addition, the Ru(II) bathophenanthroline complexes are photochemically stable so that no bleaching is observed. The sulfonate derivatives of these complexes are water soluble which makes them suitable to be used in combination with biomolecules in the aqueous phase. We have modified the complexes in such a way that they carry a single carboxyl function as to allow for couplings to amino groups of biomolecules *via* stable amide bonds.[13]

Combination of the Ru(II) bathophenanthroline complexes with suitable donor and acceptor chromophores resulted in robust resonance energy transfer (RET) systems which were applied to peptides as well as to DNA fragments providing a tool for studying intra and intermolecular interactions.[14–19] Recently, we have inserted the complexes as a relay chromophore in a three colour FRET system.[20]

The interest in genomic science has recently been shifted from DNA to proteins. A common tool in proteomics is two-dimensional gel electrophoresis to resolve the pool of individual proteins of cells. The best method for visualization of proteins in such gels is staining with Ru(II) tris(bathophenanthroline disulfonate) complexes.[21,22]

Even low abundance proteins can be detected due to the effective unspecific noncovalent associations of the Ru complexes to proteins and the high sensitivity. A further advantage of this staining technique is the fact that the complexes do not interfere after extraction of the proteins with the analysis and the identification of the proteins by mass spectrometry. In comparative studies we had demonstrated, that our Ru(II) bathophenanthroline complex **1**, which bears in addition to two sulfonylated bathophenanthroline ligands a third phenanthroline ligand equipped with a carboxyl function for attachment to biomolecules, behaves exactly like the commonly used Ru(II) tris(bathophenanthroline disulfonate) complex as far as staining sensitivity in protein gels is concerned.‡

Due to the favourable properties of the Ru(II) bathophenanthroline complexes it would be highly desirable to label proteins with them in a chemoselective reaction. They would be very

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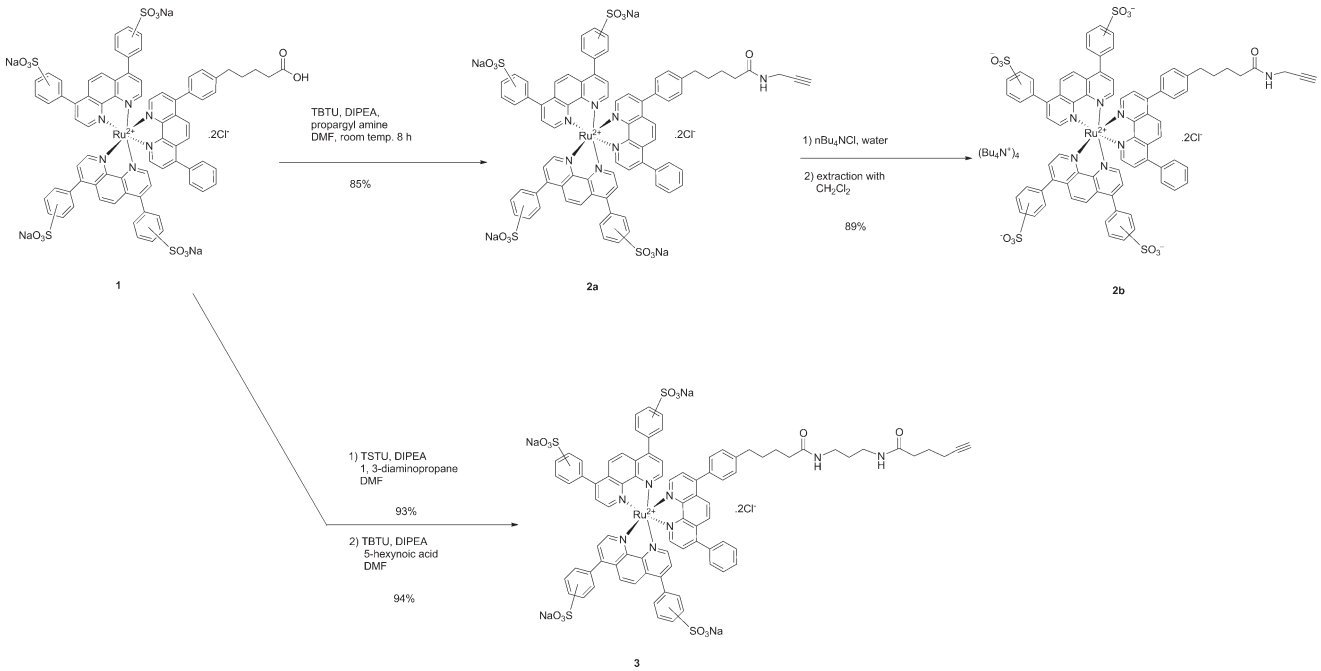
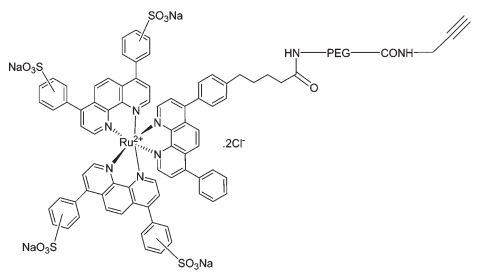
†Electronic supplementary information (ESI) available: Synthesis of various ruthenium(II) complexes used and methods for labelling of peptide and proteins are described. See DOI: 10.1039/c2ob06684j



Scheme 1 Syntheses of Ru(II) bathophenantroline complexes **2a**, **2b** and **3**.



Scheme 2 Ru(II) bathophenantroline complex **4**.

useful as very sensitive and robust reporter systems in biochemical assays. In addition, they would allow for the construction of very efficient FRET systems to study folding phenomena or interactions of two proteins. Furthermore, they would be suited to study the transport of electrons in proteins. The question is whether these efforts would be hampered by the unspecific binding characteristics of the complexes to proteins.

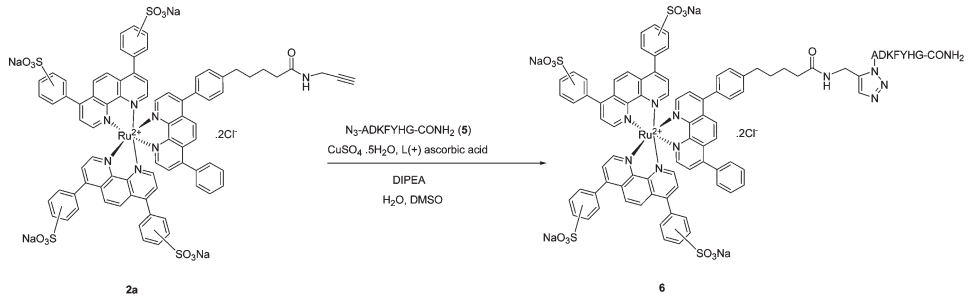
Results and discussion

In order to obtain suitable alkyne-modified Ru(II) bathophenantroline complexes for the envisaged chemoselective [3 + 2]-cycloaddition with a protein carrying an azido function our parent complex **1** was first reacted with propargylamine according to Scheme 1 to yield complex **2a**. In order to enhance the solubility of this complex in organic solvents it was also transformed into its tetrabutylammonium salt **2b**. In addition, complex **3** with an increased spacer length was prepared to see whether it would have an influence on the coupling efficiency. In preliminary investigations it had turned out that alkyne-modified PEG-derivatives were very efficient in click reactions with azido-functionalized proteins. For this reason we prepared also Ru complex **4** modified by an alkyne *via* a PEG spacer (Scheme 2).

We envisaged the use of alkyne conjugates of Ru(II) bathophenanthroline complexes for the site-specific labelling of proteins containing an azide moiety *via* the Cu^{+}-mediated click reaction. In order to validate click reactions involving Ru(II) bathophenanthroline complexes we started off with the specific labelling of a peptide containing an azide functionality at the N-terminus with complex **2a**.

The azido-modified peptide **5** was prepared by the Fmoc/tBu strategy on a resin equipped with an amide linker and using TBTU as coupling reagent.[23] While still attached in the side chain-protected form to the support, the N-terminal amino group of alanine was converted to an azide using NaN_3–Tf_2O.[24] Treatment with TFA in the presence of a scavenger for deprotection, removal from the support and precipitation gave the desired peptide in a yield of 93%. The click reaction between this peptide and the alkyne-modified Ru-complex **2** was then performed according to Scheme 3. A LC-MS of an aliquot of the reaction mixture indicated complete conversion into the labelled peptide **6**.

As a first example of a protein to undergo the chemoselective reaction between an incorporated azide function and the alkyne-modified ruthenium(II) complexes we selected the SecB protein. The protein was expressed as an azido protein containing *p*-azido phenylalanine at amino acid position 156 followed by a C-terminal HisTag ($SecB_{AzPhe156}His$) using an *E. coli*-based cell free orthogonal translation system which was depleted from termination factor RF1[25] and supplemented with enriched fractions of amber suppressor tRNA and aminoacyl tRNA synthetase specific for *p*-azido phenylalanine.[26] The system allowed for the efficient site-specific incorporation of *p*-azido phenylalanine by suppression of the stop codon TAG (amber) encoded at the C-terminus of the open reading frame of the protein gene. At the



Scheme 3 Labelling of peptide **5** with Ru(II) bathophenanthroline complex **2a** using click reaction.

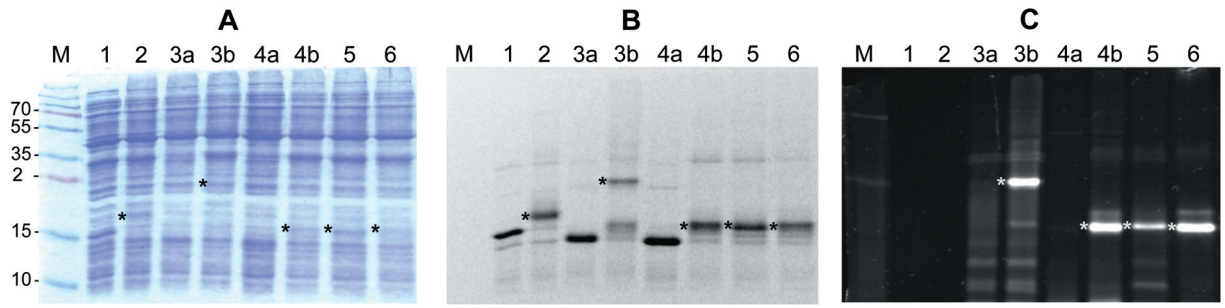


Fig. 1 SDS-PAGE analysis of click reactions of cell-free synthesized proteins namely radio labelled SecB and $SecB_{AzPhe156}His$ with alkyne-functionalized ruthenium complexes and PEG. A: Coomassie stained SDS gel, B: autoradiograph, C: fluorescence image of the SDS gel at 302 nm excitation directly after the run, *: positions of modified azido protein molecules are marked with asterisks. lane 1: cell-free expressed proteins with synthesized azido protein ($SecB_{AzPhe156}His$) before click reaction, lane 2: reaction with azido protein of lane 1 with an alkyne-functionalized PEG2000, lane 3a: reaction of protein SecB with 4, lane 3b: reaction of $SecB_{AzPhe156}His$ with 4, lane 4a: reaction of SecB with 2a, lane 4b: reaction of $SecB_{AzPhe156}His$ with 2a, lane 5: reaction of $SecB_{AzPhe156}His$ with 3, lane 6: $SecB_{AzPhe156}His$ with 2b, lane M: molecular weight marker (SeeBlue® Pre-Stained Standard, Invitrogen).

same time, newly synthesized proteins were radioactively labelled by incorporation of ^{14}C-leucine to detect and quantify them independently from attached dyes.

After incubation, the protein synthesis reactions were desalted and submitted to a click reaction with Ru-complexes **2a**, **2b**, **3** and **4** using conditions described in the supporting information.† The protein mixture was then separated by SDS-PAGE and fluorescent proteins were detected on a UV table (Fig. 1c). Excitation at 302 nm revealed a strong fluorescence of those bands containing azido proteins modified with the ruthenium dyes (Fig. 1c: lanes 3b, 4b, 5 and 6). As a negative control, the same protein was synthesized in the absence of the compounds necessary to incorporate the azido amino acid and incubated with the click reagents and the alkyne ruthenium dyes. This protein without azido amino acid could not be detected *via* fluorescence (Fig. 1c: lanes 3a and 4a), although the protein was detected with comparable yield in the autoradiograph (Fig. 1b) by means of a radioactively labelled amino acid that was incorporated during cell-free synthesis. These results demonstrate that a selective fluorescence labelling and detection of proteins within complex protein mixtures is possible using chemoselective reactive ruthenium dyes.

All four Ru(II) complexes used in the study reacted exclusively with the azido-modified $SecB_{AzPhe156}His$. No reaction with the wild type protein SecB could be observed. As far as the efficiency of the coupling is concerned, no difference could be seen applying either the sodium salts or the tetrabutylammonium salts of the Ru(II) complex (**2a** or **2b**). It was estimated to be in the range of 50% as deduced from quantification of radioactive bands in the autoradiograph. An elongation of the linker arm between the Ru(II) complex and the reacting alkyne entity as in **3** and **4** had no beneficial effect on the coupling efficiency.

To demonstrate the versatility of the approach, we have expressed four additional proteins with an azido function and submitted them to the cycloaddition with ruthenium(II) complex **2a**. The results are depicted in Fig. 2. They demonstrated again that a site specific labelling of azido-functionalized proteins is indeed possible with high efficiency using the alkyne-modified Ru(II) bathophenanthroline complex **2a**.

Conclusions

In summary, we have demonstrated that Ru(II) bathophenanthroline complexes allow for a site-specific and selective labelling of proteins even within a complex mixture of proteins despite their tendency to associate unspecifically with proteins. The thus obtained labelled proteins promise to be valuable tools in protein–protein interaction studies, especially so, if the interacting partner protein is labelled with a RET partner for the Ru-complex. Currently, we are pursuing the incorporation of two functional groups into a protein which can then be used for two different chemoselective reactions, for example for the selective coupling of two partners of a RET pair. Such constructs could then be applied to the study of folding processes of proteins as well as for protein–protein interaction.

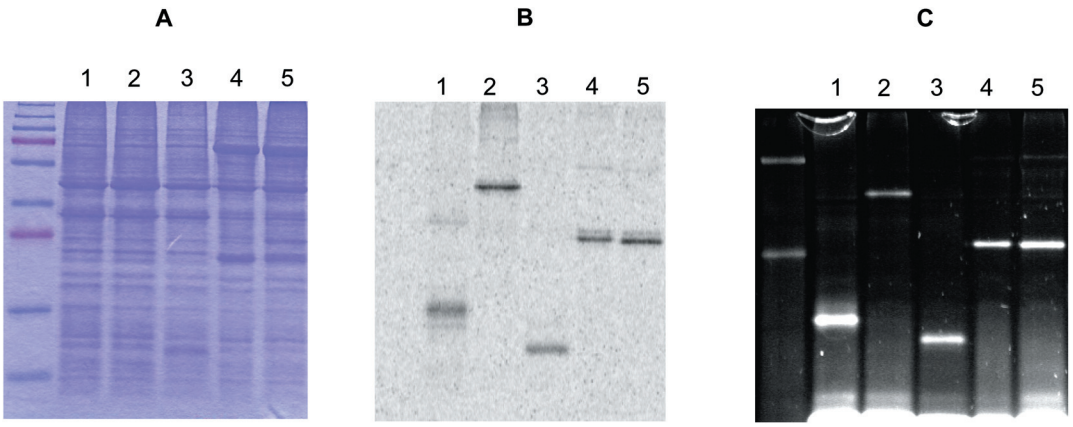


Fig. 2 Specific labeling of different azido-functionalized proteins with **2a** using click reaction in protein mixtures. A: Coomassie stained SDS gel, B: autoradiograph, C: fluorescence image of the SDS gel at 302 nm excitation directly after the run. lane1: $SecB_{AzF156}His$, lane 2: $TU\text{-}His_{AzF}$, lane 3: $HLFAR_{AzF}$, lane 4: $scFvAntiEC5218_{AzF}$, lane 5: $scFvAntiEC5218_{AzF2}$.

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