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On-Resin Assembly of a Linkerless Lanthanide(III)-Based Luminescence Label and Its Application to the Total Synthesis of Site-Specifically Labeled Mechanosensitive Channels

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A synthesis strategy for the on-resin assembly of luminescent lanthanide chelates from commercially available compounds was developed. Advantages of the approach include the absence of spacers between the metal ion and the attachment site, and the compatibility with typical chemical protein synthesis protection schemes. Methoxycoumarin-labeled lysine and tris(*tert*-butyl)-DOTA were consecutively coupled with high efficiency to a free amino group in otherwise fully protected peptide segments using standard peptide synthesis methods. Addition of stoichiometric amounts of Tb^{3+} to the modified, cleaved, and purified peptides yielded the desired lanthanide chelate. Incorporation of this label into a chemically synthesized, full-length mechanosensitive channel of large conductance (MscL) of *E. coli* and subsequent reconstitution into vesicles resulted in a functional mechanosensitive channel of comparable conductance to the wild-type channel. However, this channel required increased suction to gate. Excitation of the antenna molecule methoxycoumarin at 336 nm resulted in an emission spectrum typical for Tb^{3+} and a luminescence lifetime of 0.67 ms. The location of the probe close to the backbone of this protein may provide precise information about conformational changes during channel opening from LRET studies.

INTRODUCTION

Lanthanide(III)-based chelates are used increasingly for spectroscopy applications, in particular luminescence resonance energy transfer (LRET),[1] due to the unique luminescence properties of the chelated ions (*1*−*9*). LRET is a type of luminescence assay where a lanthanide donor (Tb^{3+} or Eu^{3+}) transfers excitation energy to an organic fluorescent acceptor in a distance-dependent manner, thus yielding static or dynamic distance information. The unique advantages of lanthanide complexes for this application include luminescence lifetimes in the millisecond range, very sharp emission spectra, high quantum yields, and, in most cases, unpolarized emission (*10*). The advantage of long-lasting fluorescence or luminescence lies in the less costly instrumentation that can be used to detect the emitted signal when compared to measurements of dyes with lifetimes in the nanosecond range. An additional advantage, in particular in the study of highly scattering solutions such as detergent micelle and liposome containing solutions, is the ability to gate the detection in order to reject stray-light. Finally, in the case of oligomeric proteins such as ion channels with multiple distances between nonequivalent positions on identical subunits, the ease of fitting emission decays of long-lived emitters to multiple decay constants allows straightforward deconvolution of these distances (*2*, *7*).

Luminescent lanthanide(III)-based chelates have been principally developed to label folded proteins or DNA. Therefore, they typically utilize flexible linkers with thiol- or amine-reactive groups, and in many cases the sensitizer itself, to distance the chelator moieties from the amino acid side chains of peptides or proteins (*1*, *3*, *11*−*14*). Whereas the presence of a spacer may be desirable for efficient labeling of folded proteins under mild conditions for steric reasons, and in cases where the bulk of the chelate label may interfere with function, such linkers may considerably reduce the accuracy of distance measurements that can be achieved through LRET by introducing additional flexibility to the system.

Our design strategy focused on the fast and facile assembly of a luminescent label that is located close to the backbone of the respective peptide or protein. The label is prepared from commercially available starting materials, DOTA as chelator and methoxycoumarine-labeled lysine as the antenna moiety in protected forms. Commercial availability of these starting materials frees the nonorganic chemist from the solution synthesis steps required for preparation of lanthanide labels described previously in the literature (*1*, *3*, *6*, *11*, *12*, *14*, *15*). The label can be introduced in a straightforward fashion during solid-phase synthesis employing either Boc or Fmoc protecting group strategies. After establishing our approach by preparing a labeled test peptide consisting of 32 amino acids using Boc-chemistry, we extended it to the assembly of multiple luminescent variants of the

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[1] Abbreviations: Acm, acetamidomethyl; CPS, chemical protein synthesis; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DPC, dodecylphosphocholine; Dpr, L-1,2-diaminopropionic acid; ESI-MS, electrospray ionization mass spectrometry; LRET, luminescence resonance energy transfer; Mca, methoxycoumarin; MscL, mechanosensitive channel of large conductance; TTHA, triethylenetetraminehexaacetic acid; TM, transmembrane domain.

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mechanosensitive channel of large conductance from *E. coli*. Loading of the DOTA chelator with Tb^{3+} was accomplished following successful synthesis and purification of the test peptide and MscL proteins, respectively, and yielded a lanthanide complex with the desired features for biophysical measurements.

EXPERIMENTAL PROCEDURES

The following reagents were obtained from the indicated sources: Boc-protected amino acids (Midwest Biotech, Fishers, IN); Fmoc-protected amino acids (Novabiochem, San Diego, CA); trifluoroacetic acid (Halocarbon, River Edge, NJ); DIEA (*N*,*N*-diisopropylethylamine) (Applied Biosystems, Foster City, CA); HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (Spectrum, Gardena, CA); acetonitrile (Burdick & Jackson, Gardena, CA). Other chemicals were obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) and were used as received.

Peptide Synthesis and Purification. Peptides were synthesized at a 0.2 mmol scale on a custom-modified Applied Biosystems 433A peptide synthesizer using S-DVB (stryrene−divinylbenzene) resin carrying an $-OCH_2-$PAM linker (Applied Biosystem, Foster City, CA) or a thioester-generating linker, respectively, following an in-situ neutralization protocol for machine-assisted Boc (*tert*-butoxycarbonyl) chemistry (*16*, *17*). Side-chain protecting groups were Arg(Tos), Asn(Xan), Asp(OcHx), Cys(pMeBzl), Glu(OcHx), His(Dnp), Lys-(2ClZ), Ser(Bzl), Thr(Bzl), Trp(CHO), and Tyr(2BrZ). The amino acid sequence of the 32mer test peptide used to prototype the DOTA-Mca-label synthesis: H-CAV-VFVTRKNRQVSANPEKKAVREYINSLELL-OH(MW: 3676 Da) was derived from the chemokine CCL5 (RANTES). The sequence and assembly of the peptide backbone of the MscL peptide segments (MscL1-55, MscL56-102, and MscL103-136) was as described previously (*18*).

Assembly of the Luminescent DOTA-Mca Label. The label was introduced on-resin into the N-terminus of the test peptide, and into the N-terminus and positions Val16 and Leu47 in the N-terminal peptide segment MscL1-55. For attachment to the N-terminus, the N-terminal Boc group was removed by treatment with 100% TFA for 2 × 1min cycles. For attachment to positions Val16 and Leu47, the side chain Fmoc protecting group of diaminopropionic acid incorporated at the desired position was removed by 3 × 3 min treatments with 3% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in DMF. Fmoc-Lys(Mca)-OH (Novabiochem, San Diego, CA) was coupled to the respective free amino group by using a 5-fold excess of the fluorescent amino acid activated with an equimolar amount of HBTU in DMF in the presence of 10% DIEA (*17*). The Fmoc protecting group on the Lys(Mca) label was removed by 3 × 3 min treatments with 3% DBU in DMF, and 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid-*tert*-butyl ester)-10-acetic acid (DOTA-tris(*tert*-butyl ester) (Macrocyclics, Dallas, TX) was coupled to the resulting free amine using HBTU/DIEA activation as described for Fmoc-Lys(Mca)-OH. The peptides were then deprotected and simultaneously cleaved from the resin support using HF (*16*). MscL peptides were purified as previously described (*18*).

Assembly of Ec-MscL. The total chemical synthesis and refolding of MscL was performed as previously described (*18*). Briefly, MscL protein was produced by consecutive native chemical ligation of three unprotected, purified peptide segments (MscL1-55, MscL56-102, and MscL103-136) to form the full-length protein. Ligations were performed at a reactant concentration of ∼0.1 mM in a solution containing 17 mg/mL dodecyphosphocholine (DPC), 8 M urea, and 100 mM NaP_i, pH 7.5, at 40 °C. Peptides were dissolved in this buffer at 40 °C for 5 min and sonicated for 3−5 min. Ligation reactions were initiated by addition of 0.5% thiophenol, and the progress of the reaction was monitored by analytical RP-HPLC and ESI-MS (electrospray ionization mass spectrometry) analysis. Prior to purification, ligation solutions were worked up at 40 °C in a 5-fold excess of a solution of 20% β-mercaptoethanol in 8 M urea containing 17 mg/mL DPC. Following purification of the first ligation intermediate MscL56-136, the Acm (acetamidomethyl) protecting group was removed from the side chain of the N-terminal cysteine residue in preparation for the final ligation step (*19*). After the final ligation, free cysteine residues were modified by adding DPC to a final concentration of >2 mg/mL to the HPLC pool and subsequent reaction with a 30-fold molar excess of bromoacetamide for 30 min at room temperature. The full-length protein containing the label was then purified. Loading of the chelator with Tb^{3+} was achieved by addition of $TbCl_3$ to the protein solution.

Electrophysiological Characterization. DOTA-Mca-labeled Ec-MscL channel protein was reconstituted into artificial liposomes as described (*18*). Single-channel recordings were made at 20−22 °C on the reconstituted labeled as well as recombinant wt protein at a bandwidth of 20 kHz at 50 mV (intracellular medium is negative) in symmetrical 250 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.1) solution. Currents were acquired with an Axopatch 200B amplifier (Axon Instruments, Union City, CA) and digitized with an Axon Digidata 1322A digitizer. Conductance recordings were analyzed using Axon pCLAMP 8 software. The probability of opening (P_{open}) vs suction curves were generated using software developed by one of the authors (G.S.). Boltzmann functions were fitted to the dose−response relations using the Microcal Origin 6.0 software, yielding activation midpoints and gradients.

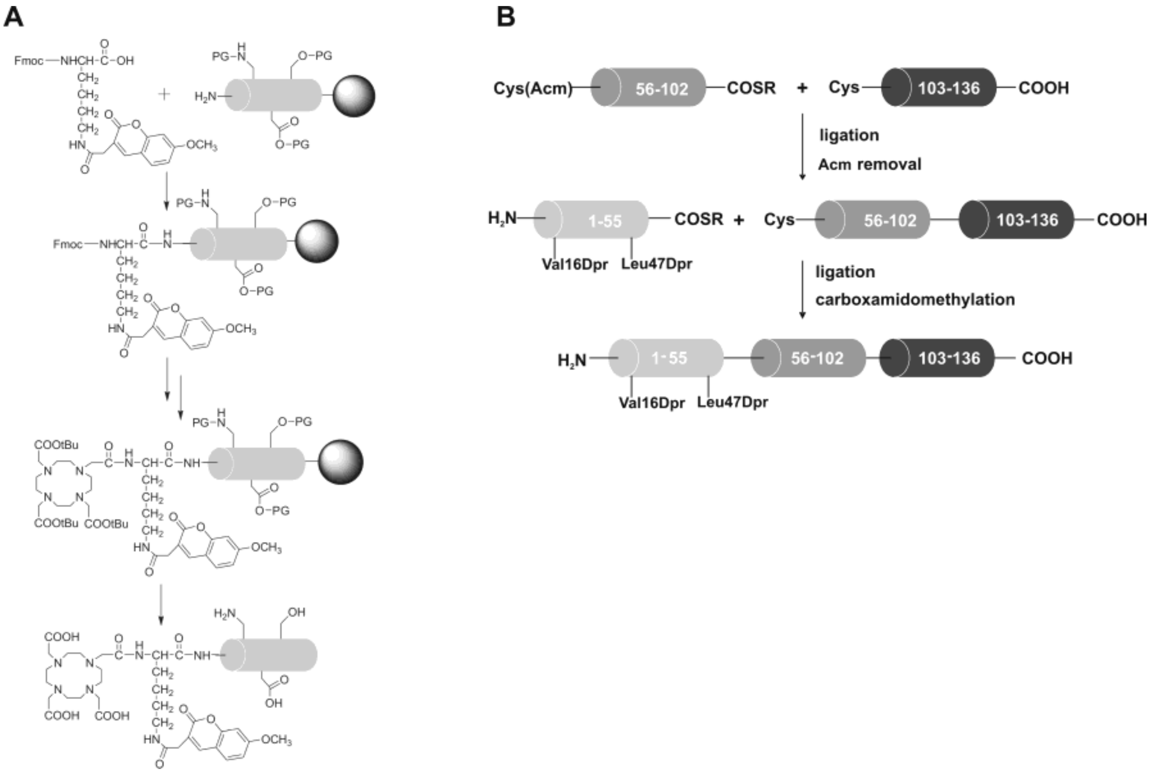
Fluorescence Spectroscopy. Fluorescence spectroscopy was performed with a FluoroLog-3 system (Jobin Yvon, Edison, NJ) equipped with a Xenon-lamp for continuous excitation and a pulsed Xenon lamp for time-resolved measurements using the phosphorimeter setup. Typical settings were an excitation wavelength of 336 nm for the Mca fluorophore, a delay of the detection window relative to the flash pulse of 50 μs, number of flashes 10 (at 20 Hz), and a scanning range of 370 to 700 nm. Data obtained from lifetime measurements were fitted with a single-exponential equation using Kaleidagraph (Synergy Software).

Mass Spectrometry. All crude, purified, and ligated peptides were identified by ESI-MS using ABI-150 or API−III mass spectrometers (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Our objective was to develop a simple and efficient procedure to label synthetic peptides and chemically synthesized proteins site-specifically with luminescent labels during solid-phase peptide synthesis. In our design, the lanthanide ion that ultimately determines the experimental distance in LRET experiments is close to the backbone of the peptide or protein since a lysine branch is employed to scaffold the attachment sites of the chelator and the antenna fluorophore (See Scheme 1). The more distant ϵ-amino group of this lysine carries the coumaryl sensitizer, while the DOTA chelator is attached

Scheme 1. (A) On-Resin Assembly of the DOTA-Mca Label; (B) Total Chemical Synthesis of Ec-MscL[a]



[a] DOTA-Mca labeling sites are indicated at the N-terminus and at amino acid positions Val16 and Leu47, respectively. Abbreviations: Acm (acetamidomethyl), Dpr (L-1,2-diaminopropionic acid).

to the proximal α-amino group. The lysine scaffold is either attached to the peptide's N-terminus or to a noncoded diaminopropionic acid residue that is incorporated into the sequence of the target peptide or protein in order to minimize the distance to the backbone. As a result, the lanthanide ion is placed at a maximum distance of 10 Å from the backbone as determined by molecular modeling. By contrast, most other luminescent chelates, such as polyaminocarboxylate−coumarin complexes, were mostly designed to employ long alkyl chains as linkers between peptide and lanthanide complex, which results in larger distances between the label and its attachment site (*1, 3, 11−14*).

The assembly of the luminescent probe was initiated by coupling Fmoc-Lys(Mca)-OH either to an unprotected α-amino group of the peptide or a side chain amino group of diaminopropionic acid in an otherwise fully protected peptide linked to a solid support (Scheme 1). Deprotection of the α-amino group was achieved by Boc removal with TFA. The side chain amino group of diaminopropionic acid was specifically deprotected by removing an Fmoc group with a sterically hindered base such as DBU. Activation and in situ neutralization using a 5-fold excess of Fmoc-Lys(Mca)-OH gave excellent coupling yields and no further optimization was needed (*17*). Subsequently, the Fmoc group was removed from the Lys(Mca) moiety by treatment with 3% DBU in DMF for 3 × 3 min and a protected DOTA was coupled to the free α-amino group. DOTA has been extensively used as a chelator for radionuclides in imaging and therapy (*20−23*). In our case, DOTA was *tert*-butyl protected at three out of its four carboxyl groups to overcome solubility problems that we encountered with unmasked DOTA in all solvents compatible with solid-phase synthesis such as DMF, DMSO, NMP, and DCM. The 4th carboxyl group was used to form a stable amide bond between the chelator DOTA and the peptide. A 3- to 4-fold excess of tris-(*tert*-butyl)-DOTA was sufficient to obtain fully modified peptides using HBTU/DIEA activation.

This synthesis strategy was initially tested on a 32 amino acid peptide by attaching the label to its unblocked N-terminus and later extended toward a C-terminal α-thioester peptide consisting of the 55 N-terminal amino acid residues of Ec-MscL. Subsequently, the purified peptide was used in the total chemical synthesis of Ec-MscL (*18*) to demonstrate compatibility with native chemical ligation strategies (see Scheme 1B). The assembly of the label gave high yields in each case. In all cases, deletion peptides missing either the coumaryl group or the DOTA group were not observed as significant side products, as determined by RP-HPLC and ESI-MS analysis of the crude cleavage products. As a result, coupling yields are estimated to be >95% overall for both coupling steps, and labeled peptides could be obtained in comparable yields to the unlabeled peptides.

Purification of the test peptide from the crude cleavage mixture by RP-HPLC was followed by lyophilization and transfer into an aqueous buffer containing 10% acetonitrile and reconstitution with $TbCl_3$. Successful complexation of the lanthanide ion by the DOTA label was verified by ESI-MS since the affinity between DOTA and Tb^{3+} was strong enough to survive the ionization process (*24*). Figures 1A and 1B show the electrospray mass spectra of the peptide before and after addition of $TbCl_3$ and desalting by RP-HPLC. The mass difference of +158 Da directly demonstrates binding of a single terbium ion to the peptide, presumably to the DOTA chelator (Figure 1B). Since excess $TbCl_3$ was removed by the desalting column, unspecific binding of Tb^{3+} to the peptide is unlikely to be the cause of the mass gain.

After this proof of principle, we proceeded to assemble labeled variants of the mechanosensitive ion channel of *E. coli* (Ec-McL). The recent total chemical synthesis of Ec-MscL allowed the direct, site-specific introduction of

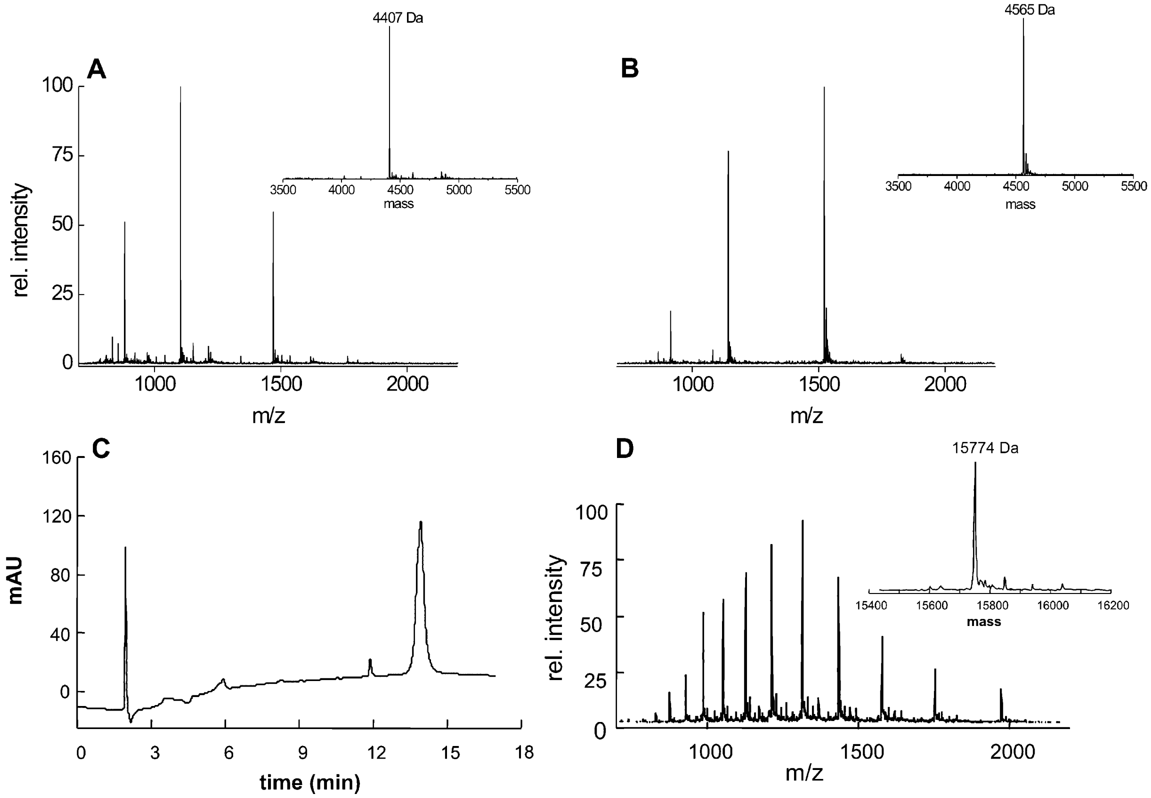


Figure 1. (A) ESI-MS of purified test peptide bearing the DOTA-Mca label (prior to Tb^{3+} addition); (B) ESI-MS of purified test peptide bearing the DOTA-Mca label (after Tb^{3+} addition); (C) RP-HPLC of purified N-terminally DOTA-Mca-labeled Ec-MscL; (D) ESI-MS of purified N-terminally DOTA-Mca-labeled Ec-MscL.

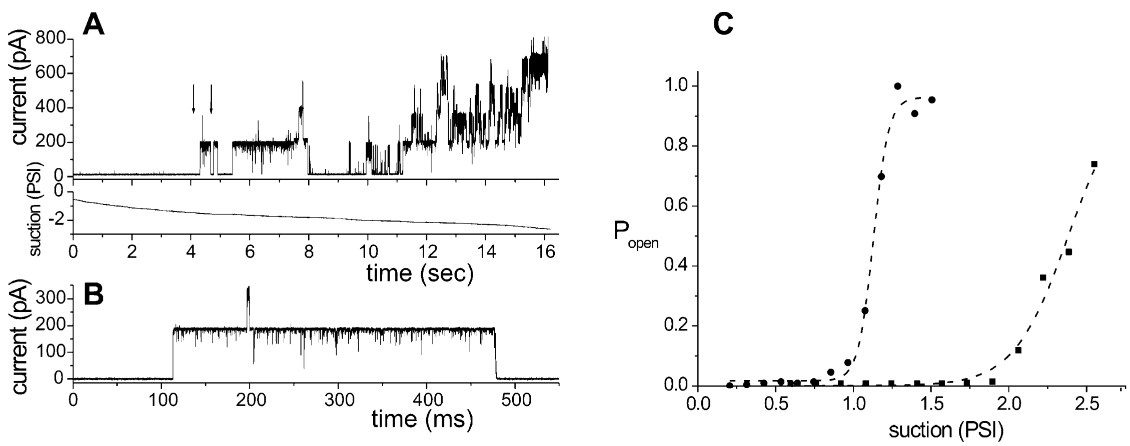


Figure 2. Electrophysiological analysis of vesicle-reconstituted N-terminally DOTA-Mca-labeled Ec-MscL. (A) Current trace (top) showing channel activity on the application of suction (bottom). The sudden increase of current at ~16 s corresponds to breakage of the patch. (B) Expanded time axis in the region of panel A indicated by arrows (suction of approximately −2.0 psi). (C) Sample opening probability curves of wt Ec-MscL (circles) and N-terminally biotinylated DOTA-Mca-labeled Ec-MscL (squares). In this experiment, activation thresholds were determined to be 1.0 psi and 2.4 psi, respectively, by using a Boltzmann fit (dashed lines).

spectroscopic labels at distinct positions of this protein during the peptide assembly stage. Since the open pore of Ec-MscL is predicted to be around 30 Å in diameter (*25*−*29*), LRET studies employing sensitized lanthanide chelates that exhibit large Förster distances and are typically most sensitive for distances between 20 and 100 Å (*8*) are uniquely suited to monitor conformational changes associated with channel opening and are not limited by the small dynamic range of EPR studies (*28*, *30*). Accordingly, a single lanthanide chelate was incorporated into three distinct positions of Ec-MscL, the N-terminus, position Val16 at the cytoplasmic end of the first membrane spanning helix of MscL, and position Leu47 in the loop region between TM1 and TM2 (Scheme 1B). Three positions were chosen in order to get a set of distances in LRET studies after transfer of luminescence to an appropriate acceptor such as TAMRA (tetramethylrhodamine).

After assembly and labeling of the respective N-terminal peptides of Ec-MscL, purification from the crude cleavage mixtures was achieved by RP-HPLC and yielded pure peptides that were used for initial testing of the fluorescent and luminescent properties of the synthetic molecule (data not shown) and as starting materials for the assembly by native chemical ligation. Yields from a synthesis at 0.2 mmol scale on the peptide level were typically 15−20 mg for unlabeled and 10−15 mg for labeled peptide. Assembly of the full-length channel protein largely followed the published procedures (*18*). The assembly process was closely monitored by RP-HPLC and ESI-MS and was performed in the absence of lanthanide ions. Chemical protein synthesis by native chemical ligation, modification of cysteine ligation sites by bromoacetamide to mask the unnatural cysteine residues, and Acm removal from the ligation intermediates did not modify or alter the label, suggesting that it is fully compatible with the chemical synthesis approach.

Figures 1C and 1D show the HPLC trace and ESI-MS of purified N-terminally DOTA-Mca labeled Ec-MscL (experimental mass: 15 774 Da (±2 Da), theoretical mass: 15 772.9 Da) after assembly (*31*). All other variants could be prepared with comparable purity, and about 1 mg of each variant was prepared in this fashion. The ESI-MS spectra of the intermediate and the final construct also demonstrate that the *tert*-butyl group of the chelator is completely removed prior to protein assembly.

The labeled channel proteins were characterized electrophysiologically in order to ensure correct folding and function. Reconstitution of the synthesized ion channel into artificial liposomes was performed as previously described (*18*) and yielded patches with resistances in the range of 5−20 GΩ. Application of suction produced characteristic single-channel activity that disappeared upon suction release. Figure 2A shows sample activity of the synthesized channel. The single-channel conductance and presence of multiple substates (Figure 2B) correspond to that of the recombinant channel (*18*); however, greater suction was required to open the DOTA-Mca-labeled channel. Figure 2C compares dose−response (P_{open} vs suction) curves for the synthetic (squares) and recombinant (circles) channels. Boltzmann fits to the dose−response curves yielded average activation thresholds of 1.3 ± 0.1 psi (9 ± 1 kPa) for wt and 2.4 ± 0.2 psi (17 ± 1 kPa) for the synthetic channel. It should be noted, however, that the higher activation threshold of DOTA-Mca-labeled channel was similar to the suction at which the liposome membrane in the patches regularly ruptured. Thus, the reported activation midpoint for the DOTA-Mca-labeled channel gives a lower boundary estimate of the value.

Overall, the electrophysiology data confirm that the described synthesis and ligation steps yielded the properly folded and functional protein. However, the introduction of the N-terminal DOTA-Mca label seems to alter channel function either by interfering with the transfer of tension from the bilayer to the channel or by stabilizing the closed-state or destabilizing the open-state. Others have also observed that modifications to the N-terminus can affect the pressure sensitivity of Ec-MscL (*32*). Unfortunately, no reproducible MscL-like conductance could be measured for the MscL variants labeled at positions Val16 and Leu47. In hindsight, this could be rationalized by the location of the label at the membrane−water interface, which might be important for transducing mechanical tension into protein conformational change (*33*). The exploration of alternative labeling sites is ongoing.

To assess the luminescent properties of the resulting protein constructs after reconstitution into a TFE/water mixture (4:1), the methoxycoumarin ring of the label was excited at 336 nm, inducing a strong fluorescence with a maximum intensity at 393 nm due to spontaneous fluorescence of the sensitizer. In the absence of lanthanide ions such as Tb^{3+}, no luminescence signal could be detected. However, after additon of an aqueous solution of $TbCl_3$ to this solution, Tb^{3+} luminescence was detected (Figure 3A). This emission was partially hidden by the stronger Mca background fluorescence. When

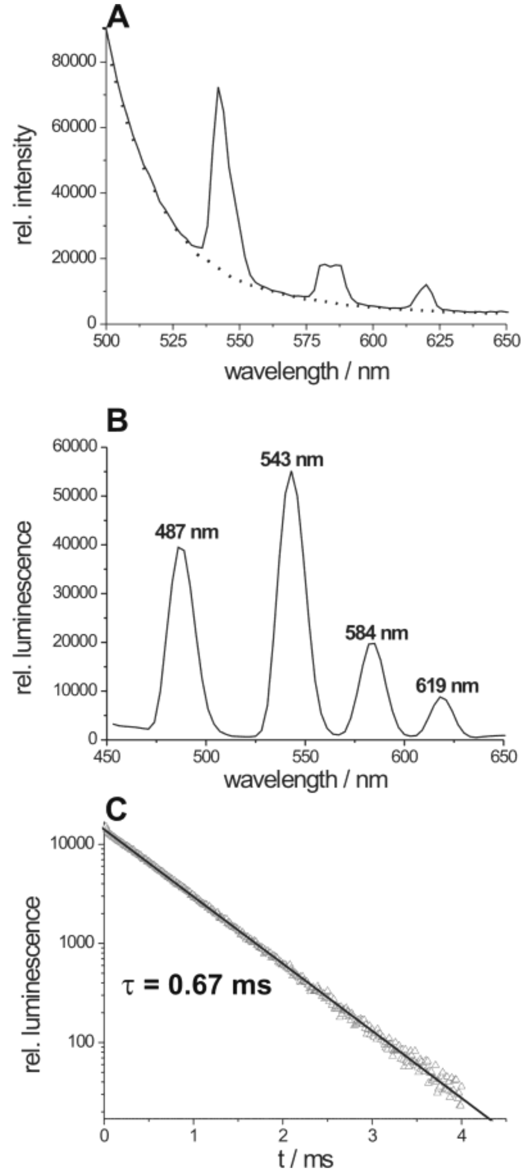


Figure 3. Luminescence of DOTA-Mca-labeled Ec-MscL after excitation at 336 nm. (A) Emission of DOTA-Mca-labeled Ec-MscL with (solid line) and without (dashed line) Tb^{3+} after CW excitation and detection; (B) Tb^{3+} luminescence, after pulsed excitation with a 10 μs pulse and gated detection (50 μs delay); (C) semilogarithmic plot of Tb^{3+}-luminescence decay.

using a phosphorimeter set up that allowed time-gated measurements, a 50 μs delay between excitation of the sample and the starting point for detection of the emission was sufficient to obtain a completely independent Tb^{3+} luminescence signal (Figure 3B). The Tb^{3+} emission showed typical, sharp maxima at 487, 543, 584, and 619 nm, with the strongest emission around 543 nm. The decay was monoexponential, and the luminescence lifetime was determined as 0.67 ms from a semilogarithmic plot of the decreasing luminescence intensity. 0.67 ms is about half of the lifetime found for DOTA-carbostyril-based Tb^{3+} chelates ($\tau \sim 1.5$ ms) in solution (Figure 3C) (*34*). The reduced lifetime may be attributed to less efficient shielding of the Tb^{3+} ion when bound to its DOTA chelator in the context of this chelate. The coordination of water, which might result in nonradiative de-excitation processes, might be facilitated in this chelate. The intrinsic quantum yield for the terbium chelate was estimated by taking the ratio of the luminescence lifetime of Tb^{3+} in the DOTA-Mca chelate and the intrinsic lifetime of Tb^{3+} (4.75 ms) (*35*, *36*). The resulting estimated luminescence quantum yield of DOTA-chelated terbium in our label is thus 14%. More detailed studies may be needed to investigate the cause of the reduced lifetime and provide a more exact luminescence quantum yield. Nonetheless, the observed luminescence lifetime of the DOTA-Mca chelate is sufficient for LRET experiments.

CONCLUSIONS

We have demonstrated that membrane proteins, site-specifically labeled with a luminescent lanthanide(III)-based chelate, can be prepared by chemical protein synthesis. Preparation of the sensitized chelate from commercial starting materials is a significant improvement to previously reported labels. Introduction of the label close to the protein backbone may allow highly precise measurements of distance changes during the opening of mechanosensitive channels by luminescence resonance energy transfer. Taken together, CPS and LRET provide a powerful combination of techniques for studying the structure−function relationship of membrane proteins such as Ec-MscL.

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