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Isothiocyanatoluminol as a Chemiluminescence Labeling Reagent for Amino Acids and Proteins

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Isothiocyanatoluminol (ITL) was successfully synthesized from the reaction between luminol and thiophosgene in the presence of triethylamine. The optimized conditions were extended for the preparation of isothiocyanatoisoluminol (ITIL) and phenyl isothiocyanate (PI). The chemiluminescence (CL) of ITL was comparable to luminol. ITL was used as a CL labeling reagent for amino acid and bovine serum albumin (BSA) in which the $-NH_2$ terminus of amino acid residue present in BSA acts as the labeling site.

Due to the minute amount of analytes present in biological fluids, sensitive and selective detection is highly desired.[1,2] In the past decade, there have been a number of analytical techniques available for sensitive detection, e.g., mass spectrometry, liquid chromatography, capillary electrophoresis, immune reaction, fluorescence, and chemiluminescence (CL). In particular, CL has gained research attention due to the fact that it provides the detection of biological samples with good sensitivity and selectivity.[3–5]

CL is the process in which the chemical reaction of CL compound raises its energy to the excited state and, upon returning to the ground state, releases energy in the form of light. Luminol (L) and isoluminol (IL) are well-known CL reagents in which the mechanism for CL emission is extensively studied.[6–8] CL compounds have been widely used in chromatographic techniques as a labeling reagent. In general, a CL labeling reagent consists of two reactive functionalities; i) chemiluminophore and ii) reactive functional group which serves as the attaching (labeling) site for the analyte. In order to offer (iso)luminol as CL labeling reagents, the chemical modification of their structures is required. However, chemical modifications of (iso)luminol structures generally lead to the loss of their CL capability.[9] Isothiocyanatoisoluminol (ITIL) has already been synthesized and is commercially available with a comparable degree of CL emission to isoluminol.[10] Due to the high quantum efficiency of luminol, isothiocyanatoluminol (ITL) is highly desired (Figure 1).

ITL was successfully synthesized from the reaction between luminol and thiophosgene in the presence of triethylamine. Synthetic conditions for ITL were also employed for the preparation of ITIL and phenyl isothiocyanate (PI). The kinetic profiles for CL emission of ITL and luminol were evaluated and found to be comparable (Figure 2). ITL was used as a CL labeling reagent for amino acids and proteins.

Initially, ITL was synthesized from the reaction between luminol and thiocarbonyldiimidazole (TCI) in the presence of triethylamine as base (Table 1). Various organic solvents and reaction times were investigated. Unfortunately, the starting

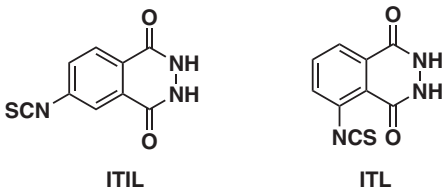


Figure 1. Chemical structures of isothiocyanatoisoluminol (ITIL) and isothiocyanatoluminol (ITL).

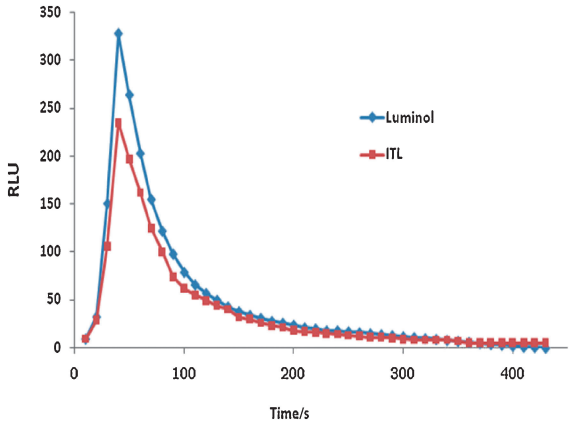
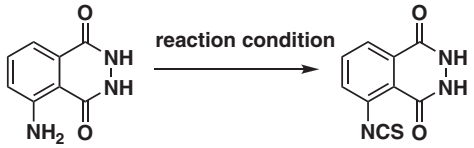


Figure 2. Time-course for CL emission of ITL and luminol.

Table 1.[a]



L ITL

Entry	Reagent	Solvent	Time /h	Yield /%[b]
1	TCI	THF	6	0
2	TCI	DMF	6	5
3	TCI	DMF	12	5
4	TCI	DMF	24	5
5	Thiophosgene	DMF	6	30
6	Thiophosgene	DMF	12	45
7	Thiophosgene	DMF	24	55

[a]Reactions were performed with 1 equiv luminol, 1 equiv Et_3N, and 1 equiv TCI or thiophosgene in corresponding solvents under nitrogen atmosphere at room temperature. [b]Based on isolated product.

material, luminol, was mainly recovered. This could be due to the low reactivity of TCI where imidazole is not a very good leaving group ($pK_a \approx 6.99$) compared to the halide substituent.[11,12] *N*,*N*-Dimethylformamide (DMF) was found to be the optimized solvent offering a high solubility of luminol. To improve the reactivity of reagent, highly reactive thiophosgene was used instead of TCI. After the reaction proceeded for 24 h, a yellow product was obtained in moderate yield (Table 1). Its molecular mass was identified by high-resolution mass spectrometry and found to correspond to the molecular weight of ITL [m/z 220 ($M + H^+$)].

It is known that chemical manipulation of (iso)luminol structures deteriorates their CL efficiency.[9] Therefore, CL emission of ITL was checked against the starting material. The CL emission obtained from ITL was comparable to luminol indicating that the introduction of isothiocyanate group into luminol did not compromise CL efficiency.

In order to ensure that these synthetic conditions could be used for the introduction of isothiocyanate group into other amine compounds, the synthetic method was employed for the preparation of ITIL and phenyl isothiocyanate. After completion, ITIL and phenyl isothiocyanate were obtained in moderate 62 and 44% yields, respectively. This demonstrated the efficiency of the protocol for the installation of isothiocyanate groups into primary amines.

In order to employ ITL as a labeling reagent for various proteins in the supersensitive CL assay, it is necessary to ensure that ITL could be used as a labeling reagent for amino acids. Since all proteins possess various amino acids which are abundant with $–NH_2$ terminus on their surfaces, the $–NH_2$ terminus is expected to serve as a labeling site for ITL. To test this hypothesis, 10 representative amino acids were used for CL labeling with ITL. The labeling of amino acids with ITL was simply achieved via the incubation of the mixture between a corresponding amino acid and ITL at 60 °C for 2 h before analyzing the product by mass spectrometry. It was found that all amino acids were successfully labeled with ITL (Table 2). The labeling mechanism is achieved via the nucleophillic addition of the $–NH_2$ group of the amino acid to the isothiocyanate group of ITL.

Since ITL could be successfully employed as a CL labeling reagent for amino acids, we targeted the ITL-labeled protein. Bovine serum albumin (BSA) was chosen as a model protein since it is widely used in various immunoassays due to its low cost and stability.[13,14] The labeling conditions (solvent) were then optimized. A mixture of DMSO and water in a ratio of 1:9 (v/v) was chosen as the solvent since both BSA and ITL were well dissolved. The optimum temperature and incubation time were found to be 60 °C for 24 h. To confirm the presence of ITL-labeled BSA, Gel-filtration liquid chromatography (GFLC) was employed for identification. After incubating BSA and ITL for 1 h, the labeled product was analyzed by GFLC. Results showed the presence of a new peak at the retention time of 15 min which was later identified as ITL-labeled BSA (Figure 3). The intensity of this peak increased with the progression of reaction time. To further confirm the identity of ITL-labeled BSA, it was spotted on a poly(vinylidene fluoride) (PVDF) membrane prior to CL detection employing a reported protocol.[15]

Kai et al. synthesized luminol-labeled dextran probe (Dex-Lu) for the CL detection of CYP3A4 protein on a solid-phase membrane. The dextran probe is highly sensitive and able to detect CYP3A4 protein at the femtomole level.[15] The signal amplication of the dextran probe originated from the multiple incorporation of luminols onto a macromolecular dextran template. In order to compare the CL efficiency of ITL-labeled BSA, Dex-Lu which was prepared according to Kai's procedure was used as a reference. The molar concentration of Dex-Lu (0.15 μM) was estimated to be the same as ITL-labeled BSA. It was found that the CL intensity of ITL-labeled BSA increased as a function of reaction time. After the reaction was left for 24 h, the CL emission of ITL-labeled BSA on a solid-phase membrane was at its highest and comparable to that of Dex-Lu (Figure 4). Comparing to the *Mr* of dextran (7×10^5) and BSA (6.6×10^4), the result from a solid-phase CL detection demonstrated a good efficiency of ITL incorporated into the smaller BSA template. The obtained CL results on a solid-phase membrane were in accordance to GFLC data. From preliminary results, ITL was successfully synthesized and employed as an effective CL labeling reagent for amino acids and proteins.

In conclusion, ITL was synthesized under optimized conditions. The introduction of an isothiocyanate group maintained its CL efficiency. The conditions were further employed for the preparation of other isothiocyanate-containing compounds, e.g., ITIL and phenyl isothiocyanate. ITL was success-

Table 2.[16]

Entry	ITL labeled amino acid	Expected *Mr*	Found (m/z)
1	Ala–ITL	308.06	309.08
2	Arg–ITL	393.12	394.13
3	Asn–ITL	337.05	360.14
4	Cys–ITL	340.03	341.08
5	Glu–ITL	366.06	367.07
6	Leu–ITL	350.10	351.11
7	Lys–ITL	365.12	366.12
8	Met–ITL	368.02	369.07
9	Phe–ITL	384.09	385.10
10	Pro–ITL	334.07	335.08

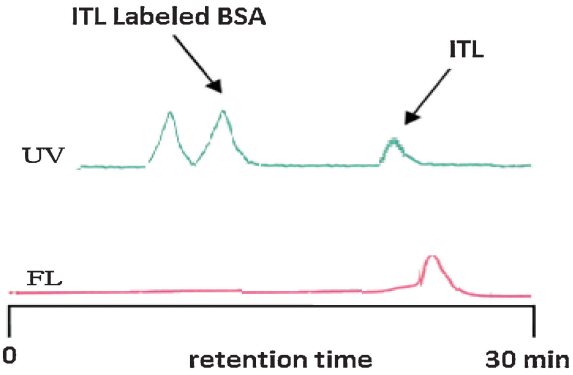


Figure 3. GFLC of ITL-labeled BSA. Conditions: injection volume, 5 μL; column, TSK gel T2000SW; eluent, 0.1% (v/v) aqueous solution of trifluoroacetic acid; flow rate, 1.0 mL min^{-1}; UV detection, lamp: 275 nm; fluorescence detection, excitation (mercury lamp)/emission (wavelength cutoff filter): 254/ >360 nm.

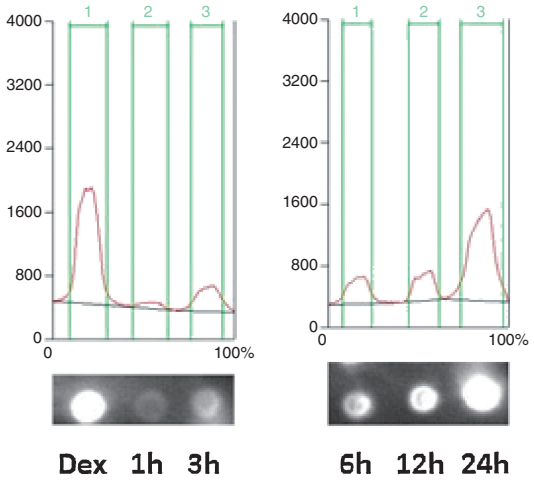


Figure 4. CL images of ITL-labeled BSA on a PVDF membrane.

fully evaluated as a CL labeling reagent for amino acids and proteins.

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