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# A novel biocompatible europium ligand for sensitive time-gated immunodetection

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**We describe the synthesis of a novel hydrophilic derivative of a tetradentate *β*-diketone europium ligand that was used to prepare an immunoconjugate probe against Giardia lamblia cysts. We used a Gated Autosynchronous Luminescence Detector (GALD) to obtain high quality delayed luminescence images of cells 30-fold faster than ever previously reported.**

Luminescence arising from the long-lived excited state of trivalent europium ions provides a valuable means to temporally resolve a labeled target. Time gated luminescence (TGL) detection relies upon a brief but intense excitation pulse followed by a short interval to allow prompt fluorescence to decay.[1] Luminescence from lanthanide labeled target molecules can then be captured free of background fluorescence to yield images with a greatly enhanced signal to noise ratio. For this reason, lanthanide chelates are of significant interest as alternatives to conventional fluorophores. In fact, detection sensitivities of $10^{-12}$-$10^{-15}$ M can be achieved with lanthanide chelates, exceeding that attainable with conventional prompt fluorophores.[2-6]

Luminescent (lanthanide) nanoparticle systems are an alternative technique for biomolecule labeling that have the potential to produce brighter labels and to deliver enhanced sensitivity in time-gated luminescence modes. Improved photostability, brightness and insensitivity to environment must be balanced against the challenges of working with complex surface chemistry used to prepare homogeneous, aggregate free and soluble immunoconjugates.[7, 8]

Since the first report on the application of fluorescent labels in immunohistochemistry by Coons *et al.*,[9] this field of science has evolved immensely. Nonetheless, almost all immunoassays suffer to some extent from the problem of background signal arising from sample auto-fluorescence, which limits the sensitivity of detection. Time-gated luminescence probes largely eliminate the problem of auto-fluorescence to make TGL detection among the most sensitive fluorescence method known.[10, 11]

Attachment of luminophores to an antibody can be achieved either by direct labeling of antibodies or by indirect labeling of secondary detection reagents, such as streptavidin (for a biotinylated antibody), bovine serum albumin (BSA) or thyroglobulin (TG).[12-15]

Unlike organic fluorophores, lanthanide conjugated bio-molecules are not susceptible to self-quenching. As a result high signal amplification can be achieved by multiple labeling of the biomolecule of interest, although this approach can be problematic since it often results in precipitation of labeled biomolecules. This effect is partially due to the hydrophobic character of the aromatic antenna (organic chromophore) present in lanthanide chelates. The nature of the linker that separates the ligand from the antibody can also have a profound effect on conjugate stability. It has been reported that the linker chemistry is critical in determining the stability, efficacy and specificity of the immunoconjugate.[16] We investigated this phenomenon by appending a linker moiety with the aim of improving the bioconjugable nature of BHHCT.

*β*-Diketone lanthanide chelates have found application as intense luminescent agents for roles in sensitive immunodetection. The utilization of terphenyl as the parent molecule for tetradentate *β*-diketones has yielded a range of high molar extinction coefficient (ε) sensitizers including BHHCT, BPPCT, BTBCT and BHHBCB.[14, 15, 17] A significant problem with these ligands is their poor aqueous solubility that can induce precipitation of heavily labeled immunoconjugates.[10]

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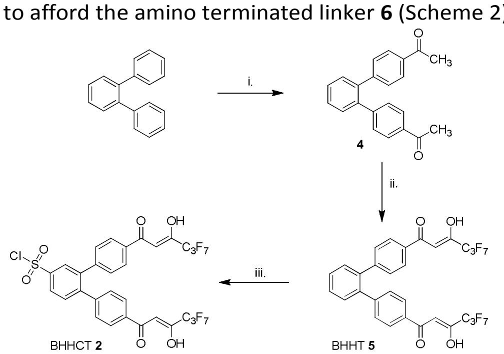
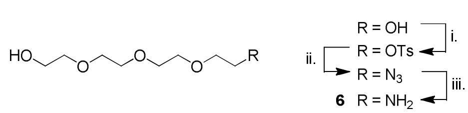
We describe here a synthetically developed biocompatible europium ligand [BHHTEGST **1** (4,4'–bis(1'',1'',1'',2'',2'',3'', 3''-heptafluoro-4'',6''-hexanedion-6''-yl)sulfonylamino-tetraethyleneglycol-succinimidyl carbonate-*o*-terphenyl)] an improved version of our previous probe BHHST **3**.[18] Aqueous solubility was enhanced by replacement of the propanyl linker present in **3** with tetraethylene glycol; both probes contain an *N*-hydroxysuccinimide ester as an activated attachment point (Figure 1). BHHTEGST was covalently attached to lysine residues of antibody (G2O3-specific for *Giardia cyst*) through the NHS group.

The synthesis of BHHTEGST **1** commenced with the preparation of BHHCT **2** according to literature methods[14] with modifications to improve the yield and purity in each step of the synthesis (Supporting Information). BHHTEGST **1** synthesis was initiated by preparation of 4,4'-diacetyl-*o*-terphenyl **4** using standard electrophilic substitution conditions between *o*-terphenyl and acetyl chloride in the presence of anhydrous aluminium chloride in dry dichloromethane (Scheme 1). This reaction was optimized by addition of a solution of *o*-terphenyl in dichloromethane in a drop-wise fashion to a mixture of anhydrous aluminium chloride and acetyl chloride in dry dichloromethane at 0 °C to give **4** in 75% yield as yellow crystals. Claisen condensation of **4** was performed by using sodium methoxide and ethyl heptafluorobutyrate in dry tetrahydrofuran to give BHHT **5** in 85% yield as a yellow powder, upon the addition of absolute ethanol. Finally, BHHCT **2** was obtained in quantitative yield by treatment of BHHT **5** with chlorosulfonic acid.

The tetraethylene glycol (TEG) linker was synthesized *via* mono-tosylation of tetraethylene glycol in 86% yield. The original procedure was improved by grinding sodium hydroxide under anhydrous conditions, followed by rapid addition to the reaction mixture. The tosyl group was then converted to an amine *via* the azide, in 77% yield over the two steps, to afford the amino terminated linker **6** (Scheme 2).[19]

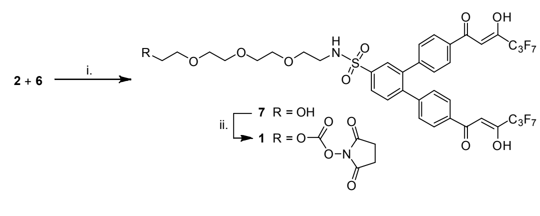


**Scheme 1.** i. $AlCl_3$, AcCl, dry DCM, 75%; ii. Ethyl heptafluorobutyrate, NaOMe, THF,85%; iii. $ClSO_3H$, 98%.



**Scheme 2.** i. NaOH, THF, *p*-TsCl, 86%; ii. $NaN_3$, EtOH, 70 °C, 81%; iii. Pd/C, $H_2$, EtOH, 95%.

The maximum yield of the immediate precursor to BHHTEGST, compound **7**, was achieved by addition of BHHCT **2** in a drop-wise fashion to a solution of **6**, DMAP and TEA in dry acetonitrile, resulting in 80% yield of **7** (Scheme 3). The final stage of BHHTEGST **1** synthesis was performed by using *N,N*-disuccinimidyl carbonate (DSC), DMAP, TEA in dry acetonitrile and the crude reaction mixture was purified *via* preparative HPLC C18 and lyophilized to give a light yellow powder of BHHTEGST **1** in 65% yield.



**Scheme 3.** i. DMAP, TEA, MeCN, 80%; ii. DSC, DMAP, TEA, MeCN, 65%.

To evaluate BHHTEGST, we used conjugated antibody probe (G2O3-BHHTEGST) to perform time-gated luminescence immunodetection in the presence of strong autofluorescence. The cysts were imaged in TGL mode on a BX51 microscope with the GALD inserted into the DIC slot of the instrument.[20] The TGL images were obtained in the shortest exposure time ever achieved, 30 to 500 fold shorter than previously reported in the literature.[21-26]

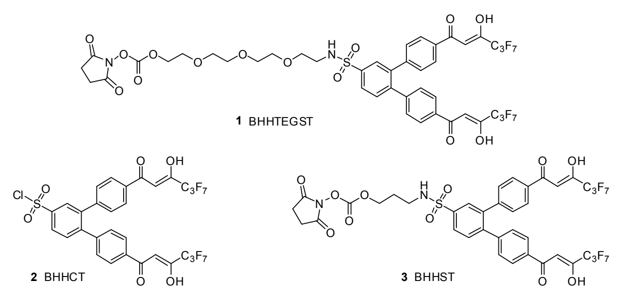


**Figure 1.** BHHCT synthetically developed derivatives (**1**: BHHTEGST; **2**: BHHCT; **3**: BHHST)

G203 immunoconjugates were prepared with commercial BHHCT and BHHTEGST over a range of Fluorescence to Protein ratios (F/P) for luminescence intensity and lifetime comparison (SI). The improved aqueous solubility of BHHTEGST over BHHCT was demonstrated following addition of identical concentrations of each ligand to a G203 protein solution. As shown in Figure S34 SI, conjugation of BHHTEGST to antibody resulted in a clear solution whilst BHHCT generated a cloudy mix of suspended colloids.

The photophysical properties of BHHTEGST were investigated and compared with BHHCT ligand. $Eu^{3+}$ chelation capacity of both BHHTEGST and BHHCT was determined by titration of ligands with $Eu^{3+}$. As shown in Figure S27 SI, the luminescence intensity for both chelates increased until 1 molar ratio of $Eu^{+3}$ to ligand was reached, implying the formation of BHHTEGST-Eu and BHHCT-Eu complexes.



Further increasing the concentration of $Eu^{+3}$ ions did not increase luminescence intensity of chelates strongly suggesting that the chelates are saturated at 1:1 ratio with $Eu^{+3}$ even with 10 equivalent molar ration of $Eu^{+3}$ ions to ligand. The formation of stable 1:1 complex of $Eu^{+3}$ ions to ligands is in accordance with a previous study on a similar diketone chelate BTBCT.[13] The luminescence spectral profile of both chelates BHHTEGST-Eu and BHHCT-Eu, displayed near identical emission profiles, suggesting that the tetraethylene glycol NHS moiety has negligible interaction with the diketone-$Eu^{3+}$ complex (Figure S29 SI).

The luminescent intensity of G203 bioconjugates over a range of F/P ratios was measured and a strong positive correlation with intensity was observed (Figure S30 SI). We plotted luminescence intensity as a function of F/P ratio with the results shown in Figure S31 SI. Considering that luminescence intensity of the bioconjugate showed a linear correlation ($R^2$ = 0.9769) to the number of ligand attached tends to confirm that luminophore moieties are not self quenching in this configuration.

We next investigated the stability of $Eu^{3+}$ chelation by the diketone moiety present in both BHHTEGST and BHHCT. Bioconjugates of each ligand were prepared with bovine serum albumin (BSA) and titrated against EDTA whilst monitoring luminescence intensity. Both BSA-BHHTEGST-Eu and BSA-BHHCT-Eu display similar stability in the presence of EDTA with luminescence intensity decreasing markedly at concentrations of EDTA higher than $1.0 \times 10^{-4}$ M; EDTA complexes the $Eu^{3+}$ and makes it unavailable to the ligands (Figure S33 SI). The results of the EDTA stability titration with BHHCT are in accordance with the previous study by Zhang, L. *et al.*[17]

To determine quantum yield we compared BHHTEGST against commercially purchased BHHCT (reported QY of 0.25).[27] The molar extinction coefficient was determined for each ligand, BHHTEGST and BHHCT (31,400 $M^{-1}.cm^{-1}$ versus 30,300 $M^{-1}.cm^{-1}$), see Table S3 SI. For each compound, luminescence intensity in water, $D_2O$ and fluorescence enhancing buffer (FEB) was measured with a gate delay of 100 µs on an Agilent Cary Eclipse Fluorescent Spectrophotometer. For each compound, luminescence intensity was lowest in water, slightly higher in $D_2O$ and significantly enhanced in FEB (3.8 fold for BHHCT and 3.0 for BHHTEGST relative to water) (Figure S35-36 SI). Excitation wavelength and refractive index of solutions were identical for each set of measurements and permit us to use a simplified equation for QY calculation:

$$\phi_x = \phi_{ref} \frac{I_{Tx}.\varepsilon_{ref}}{I_{Tref}.\varepsilon_x}$$

Where $\phi_{ref}$ is the reported QY of BHHCT, $\varepsilon_x$, $\varepsilon_{ref}$ is the molar extinction coefficient for BHHTEGST and BHHCT respectively, $I_{Tx}$, $I_{Tref}$ is the integrated emission for each chelate summed from 0-800 µs interval.Using these input parameters we arrive at a figure of 0.227 for the QY of BHHTEGST.

Luminescence lifetime (τ) is another critical parameter for consideration, BHHTEGST in FEB displayed a 27% reduction in luminescence lifetime compared to BHHCT (253 µs versus 347 µs, $R^2$ = 0.9985). Initial luminescence measurements were taken after 100 µs so we can calculate initial intensity of each ligand solution at $T_0$ using the exponential equation $I_T = I_0 e^{\frac{-T}{\tau}}$ where $I_T$ equals luminous intensity after time T and τ is luminescence lifetime (both in µs).[1] We calculate intensity at $T_0$ of BHHTEGST would be 696.3 versus 605.6 for BHHCT. The area under the exponential curve from time $T_0$ to $T_{800}$ for BHHTEGST represents 96.4% of that for BHHCT (Figure S38 SI). The photophysics leading to sensitized emission from a lanthanide ion are complex; it is often observed that small changes in antenna molecule chemistry can lead to large changes in emissive output.[29] Experimentally, we found that appending a tetraethylene glycol linker to BHHCT decreased luminescence lifetime and boosted initial brightness. From the perspective of optimizing image brightness, BHHTEGST has the advantage of being useful at higher F/P ratios than BHHCT and consequently delivers stronger signal.

BHHTEGST contains an *N*-hydroxysuccinimide ester (NHS) cross linking group which enables attachment of the ligand to antibody *via* the amine group of lysine residues. BHHTEGST was conjugated with buffer exchanged G2O3 [100 mM $NaHCO_3$, pH 8.5]. After incubation for 1 h at 37 °C the reaction mixture was purified on a sephadex G-50 column. The fractions corresponding to labelled antibody were identified based on spectrophotometer absorbance readings (280 nm and 335 nm).

To determine the optimum conjugation level of ligand to G2O3, reactions were conducted with three different molar ratios of BHHTEGST to antibody. The number of ligands per antibody molecule (fluorophore to protein ratio, or FPR) was determined on the basis of the molar extinction coefficient at 280 nm and 335 nm for each component. The ligand has strong absorption at 335 nm whereas the antibody does not; G2O3 shows strong absorbance at 280 nm. Table S1 reports the FPR for G2O3 was 10, 14 and 19 as the molar ratio of BHHTEGST to antibody was 20:1, 40:1 and 60:1, respectively. We observed that G2O3-$BHHTEGST_{14}$ has the capacity to brightly label cells whilst maintaining low background when compared to cells labelled using other conjugation levels (G2O3-$BHHTEGST_{10}$ and G2O3-$BHHTEGST_{19}$).

Imaging results: Labeled *Giardia lamblia cysts* were imaged on a DP72 camera fitted to an Olympus BX51 microscope equipped with the GALD (see SI for details). Figure 2 shows a Giardia lamblia cyst labelled with a mixed immunoconjugate (G2O3-BHHTEGST & G2O3-FITC). The average signal to noise ratio (SNR) for Figure 2A was determined to be 7.9 and 12.7 for Figure 2B, suggesting the specificity of G203 has not been impaired by conjugation with BHHTEGST. We consistently observed that the periphery of the cells appeared fainter compared to the central portion when imaged under TGL conditions. This effect has been observed when conventional epifluorescence techniques were employed and is possibly a response to a differential distribution of antigenic sites on the cyst. Figure 3 illustrates a clump of *Giardia cysts* together with strongly auto-fluorescing (bright yellow) *Synechococcus* cells. Image A was captured at ASA 200 with exposure of 100 ms, the peak brightness of the auto-fluorescing *Synechococcus* cells was about 30% brighter than the labelled cysts to give a SNR of 0.83. The same group of cells was then imaged under TGL conditions using the GALD, again with ASA 200, exposure of 1.0 second to give a SNR of 12.7.Cells are shown as imaged without image enhancement or modification of any kind, note the complete absence of background in TGL mode (SI).

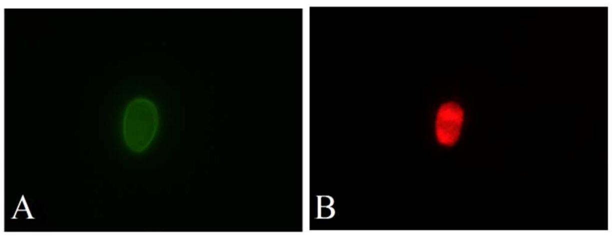



**Figure 2.** Double labelling of *Giardia cyst* cells using mix (G2O3-BHHTEGST & G2O3-FITC), (A) FITC channel (B) GALD: time-gated condition

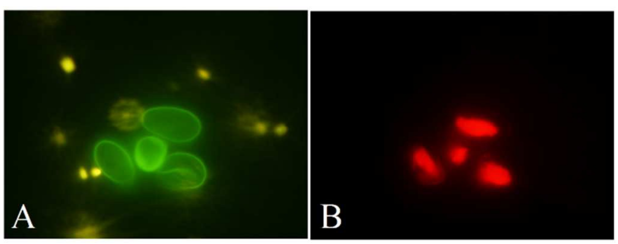



**Figure 3.** Double labelling of *Giardia cyst* cells using mix (G2O3-BHHTEGST & G2O3-FITC) (A) FITC channel (B) GALD: time-gated condition [environmental stimuli background fluorescence (*Synechococcus* cells)]. Oil immersion 100 x objective used, exposure time of 1 s.

## Conclusions

BHHCT is an easily synthesized europium ligand with good quantum yield but poor biocompatibility due to the hydrophobic characteristic typically observed for tetradentate *β*-diketone ligands. BHHTEGST displays very similar photophysical properties to the parent compound but the TEG linker affords a means to construct a biocompatible immunoconjugate probe supporting a multiplicity of luminophores. The ligand is a bright, effective label for rare cell detection and has immediate potential for the preparation of stable bioconjugates from a wide range of intermediate proteins / immunoglobulins. In conjunction with the GALD, high contrast TGL imaging was achieved with short exposure intervals. Luminous emission from the labelled cells was easily visible with the naked eye, whilst prompt fluorescence was completely suppressed; a marked improvement over all previously reported delayed luminescence imaging systems.

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