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ARTICLE TYPE

# Inter/Intramolecular Cerenkov Radiation Energy Transfer (CRET) from a fluorophore with a built-in radionuclide.

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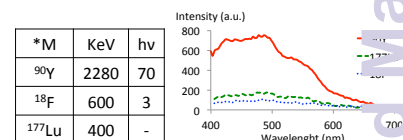
The Cerenkov Radiation (CR) from [<sup>18</sup>F]-FDG, [<sup>177</sup>Lu]-LuCl<sub>3</sub> and [<sup>90</sup>Y]-YCl<sub>3</sub> was detected and CR Energy Transfer (CRET) to several fluorophores was examined. Subsequent fluorescence emission was found to be a function of the position of absorption bands with respect to the CR peak, energy of emitted particles, radionuclide/fluorophore loading, and fluorophore brightness. A variant of the best fluorophore with a built-in radionuclide was synthesized to achieve inter- and intra- molecular CRET.

Cerenkov Radiation (CR) is the light emitted when a particle exceeds the speed of light in an insulating medium.<sup>1</sup> Such particles that may be β<sup>-</sup>, β<sup>+</sup> (and eventually α) are emitted during radioactive decay.<sup>1</sup> CR is typically the blue glow observed in nuclear reactors (Fig. 1A). It is one variant of chemiluminescence, and is one of the many ways of light production beside bioluminescence, fluorescence, phosphorescence etc. CR has a continuous spectrum in the 250-1000 nm window, but is mostly blue-weighted (300-600 nm).<sup>1,2</sup> CR is beginning to be used in biomedical applications: β<sup>-</sup>/β<sup>+</sup> emitting radiopharmaceuticals (for PET and RIT) are now to be considered bimodal by essence.<sup>3-8</sup> Hence, the potential of Cerenkov Luminescence Imaging (CLI) may be significant because it does not require external irradiation, nor does it suffer from auto-fluorescence. It is only since 2009 that CLI became possible, with the design of ultrasensitive photon imagers.<sup>3-8</sup> Chemists have been poorly involved in this area so far, but there is a need to design probes that could achieve efficient Cerenkov Radiation Energy Transfer (CRET) process, i.e. CR absorption by fluorophores,<sup>9</sup> lanthanides,<sup>10</sup> Quantum Dots (QDs)<sup>11-14</sup> and subsequent luminescence emission. In these seminal studies, the radionuclides were exogenous, and the QDs are potentially toxic.

Herein, several parameters have been carefully examined from a chemistry standpoint to optimize an intermolecular CRET: the energy of an exogenous radioactive source (i.e. energy donor), and the optical properties of the fluorophore (i.e. energy acceptor), such as the maximum absorption band compared to the CR spectrum, and both constitutive parameters of brightness (Φ<sub>F</sub> × ε), such as the fluorescence quantum yield (Φ<sub>F</sub>), and the molar extinction coefficient (ε). With the best leads, subsequent inter- and intramolecular CRET were achieved using the best fluorophore lead with a built-in /endogenous radionuclide.

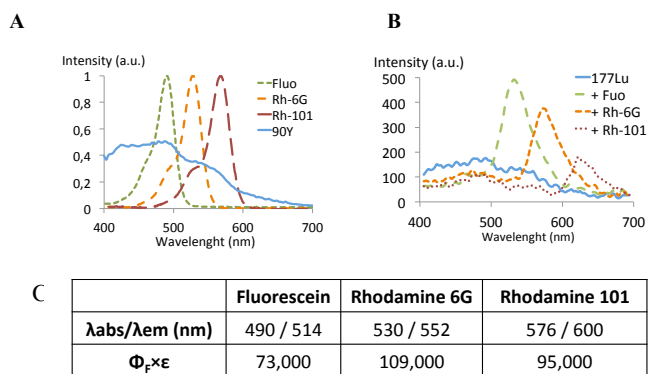
CR was detected from a solution of radionuclides: under the apparatus detection limit, CR stretches from 300 to 600 nm (Fig. S1). The intensity of the Cerenkov Radiation was examined as a function of the energy of the emitted particle (from 400 to 2280

keV) that affects its velocity, but not as a function of its charge (β<sup>-</sup> vs β<sup>+</sup>) (Fig. 1). Hence, at different radioactivity levels (9-350 MBq), it was found that the intensity of the CR increases from <sup>177</sup>Lu (400 keV, β<sup>+</sup>) to <sup>18</sup>F (600 keV, β<sup>+</sup>), and <sup>90</sup>Y (2280 keV, β<sup>-</sup>) (Fig. 1B and table). This correlates with Mitchell's simulations suggesting ca. 3 and 70 photons emitted per decay of <sup>18</sup>F and <sup>90</sup>Y nuclei, respectively.<sup>15</sup> Note that the CR intensity is also known to be a function of the refractive index η of the medium.<sup>3</sup>



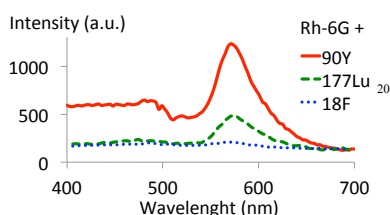
**Fig.1** A. Cerenkov Radiation (CR) observed in a Nuclear Reactor. B. Correlation between the intensity of the emitted CR, the energy of emitted β<sup>-</sup>/β<sup>+</sup> particles, the reported yield in photons per decay,<sup>8,15</sup> and the luminescence intensity. C. Spectroscopic detection of CR emitted by [<sup>90</sup>Y]-YCl<sub>3</sub> (16.2 MBq), [<sup>18</sup>F]-FDG (46.5 MBq), [<sup>177</sup>Lu]-LuCl<sub>3</sub> (318.3 MBq) in saline buffer (zoom in the 400-700 nm region).

Subsequent intermolecular CRET was examined for a constant level of radioactivity, with a series of fluorophores having comparable brightness (i.e. comparable Φ<sub>F</sub>, and comparable ε) but with absorption spectra that differ from the position of their maximum absorption bands. Our first results show the following trend, i.e. fluorophores absorbing at the CR peak emission wavelength (i.e. 495 nm) undergo intense fluorescence emission. On the other hand, the fluorophore emission intensity decreases when the fluorophore λ<sub>max</sub> shifts away from the CR peak (i.e. the overlap is less). Hence, the intensity of fluorescence goes down from fluorescein (λ<sub>max</sub> 495 nm, i.e. perfect match with CR peak emission wavelength), rhodamine 6G (λ<sub>max</sub> 528 nm, ca. 30 nm from CR peak), to rhodamine 101 (λ<sub>max</sub> 560 nm, ca 60 nm shift). Other fluorophores the λ<sub>max</sub> of which are on the edge of the CR (in the Agilent Cary-Eclipse fluorimeter detection limit), undergo either a limited emission or no emission at all (Table S5). Such a fluorophore is porphyrin TPPS (λ<sub>ex</sub> 420 nm), which emits at ca. 660 nm (Fig. S1). The CRET ratios were calculated (see SI) by using Piwnica-Worms method<sup>11</sup> that was adapted from methods developed with FRET/BRET.<sup>17-19</sup>



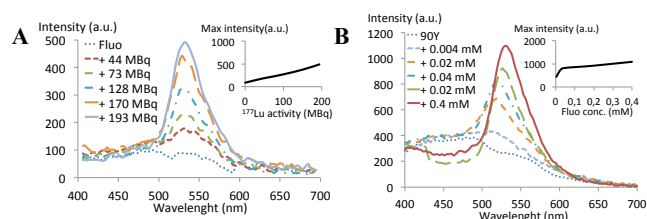
**Fig. 2:** **A.** Position of the absorption  $\lambda_{max}$  of fluorophores with respect to the CR emission spectrum. **B.** Fluorescence emission of fluorophores (0.4 mM) upon CRET (and remaining CR radiation remaining at the bottom of the spectrum) ( $^{177}\text{Lu}$  180-190 MBq). Working solution: 0.15M (0.9%) NaCl solution / MeOH (or 0.1M NaOH solution) 6:4 vol., at RT (see SI).

As shown for the CR, the resulting CRET is proportional to the flux of photons, i.e. the energy of the radionuclide, which for a given radioactivity goes up from  $^{177}\text{Lu}$  ( $\beta^+$ , 400 keV),  $^{18}\text{F}$  ( $\beta^+$ , 600 keV) to  $^{90}\text{Y}$  ( $\beta^-$ , 2280 keV) (Fig 1B). Under comparable levels of radioactivity, the amount of photons delivered to the fluorophore vary considerably from one radionuclide to another: so does the resulting CRET emission (Fig. 3), and hence the CRET ratios (tables S3, S4, S7).



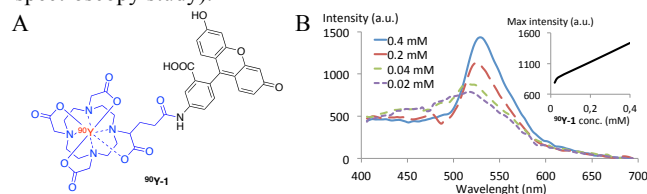
**Fig. 3:** The intensity of the Rhodamine 6G (0.4 mM) fluorescence emission is a function of the energy of the particle emitted during radionuclide decay: 400 keV ( $^{177}\text{Lu}$ , 180 MBq), 600 keV ( $^{18}\text{F}$ , 61.5 MBq), 2280 keV ( $^{90}\text{Y}$ , 9.5 MBq), i.e. the amount of emitted optical CR photons.

When the radioactivity of a given radionuclide, such as  $^{177}\text{Lu}$  drops from 350 MBq to 50 MBq, the intensity of fluorescence emission drops accordingly (Fig. 4A). A linear relationship was observed between the number of MBq and the intensity of luminescence (AU) (insert Fig. 4A). The detection limit was 50 MBq for  $^{177}\text{Lu}$  (and 1 MBq for  $^{90}\text{Y}$ ). When no radioactivity was used, no fluorescence was observed. Similarly, the intensity of fluorescence was linearly dependent upon the concentration of fluorophore added (detection limit 10  $\mu\text{M}$ ) (Fig. 4B and insert).



**Fig. 4:** **A.** Increase of the intensity of the fluorescence emission signal of fluorescein (0.4 mM) is a function of the quantity of [ $^{177}\text{Lu}$ ]- $\text{LuCl}_3$  (i.e. radioactivity). Insert: monitoring of the increase at  $\lambda_{em}$  of fluorescein (526-529 nm). **B.** Increase of fluorescence upon addition of fluorescein at constant activity (6.9-9.5 MBq). Insert: fluorescence monitored at maximum intensity (508-526 nm) upon addition of [ $^{90}\text{Y}$ ]- $\text{YCl}_3$ .

Among the fluorophores mentioned above, fluorescein was found to be the best lead because its  $\lambda_{max}$  matches the most intense peak of the CR spectrum, and hence the overlap between the two spectra is maximized. Hence, it was appended with a radionuclide to afford **1** (Fig. 5A). Target **1** was synthesized upon reaction of 5-aminofluorescein with DOTAGA-anhydride to afford the fluorescein-DOTA conjugate **1** (Fig S5). Metallation of **1** with non-radioactive  $^{89}\text{YCl}_3$  was achieved to optimize the metallation conditions and to fully characterize the  $^{89}\text{Y}$ -DOTA-fluorescein complex  $^{89}\text{Y-1}$  ( $^1\text{H-NMR}$ , HRMS: Fig. S15-16). The same conditions were applied for the reaction with [ $^{90}\text{Y}$ ]- $\text{YCl}_3$  to afford the radiolabeled complex  $^{90}\text{Y-1}$ , which was monitored by radio-TLC (Fig. S8). Subsequent fluorescein fluorescence emission was observed at pH 8, which was the result of both inter- and intramolecular CRET processes (a pure intra-molecular CRET process would require a single-molecule fluorescence spectroscopy study).



**Fig. 5:** **A.** Structure of  $^{90}\text{Y-1}$ : a fluorescein-DOTA conjugate with a built-in radionuclide (synthesis in Fig. S5). **B.**  $^{90}\text{Y-1}$  Fluorescence emission upon inter- and intramolecular CRET processes at various concentrations (and constant activity, 9 MBq). Insert: monitoring of the increase at  $\lambda_{em}$  of fluorescein (520 nm).

In conclusion, this study presented a use of the optical light emitted by radionuclides, the Cerenkov Radiation (CR), which is blue-weighted (300-600 nm). The yield in photons is a function of the energy of the radionuclide,  $^{90}\text{Y}$  is more energetic (2280 keV) hence more luminescent than  $^{177}\text{Lu}$  (400 keV). CR Energy Transfer (CRET) onto fluorophores was achieved with subsequent fluorescence emission: these processes were found to be a function of: a) the drug/radionuclide loadings, b) the energy of the emitted particle (i.e. that addresses its velocity and hence its yield in photons), and c) a good match between the maximum absorption band of the fluorophore and the CR peak emission wavelenght. Such CRET studies relied on substantial

concentrations of fluorophores, with radionuclide/fluorophore molar ratios up to 1:10<sup>6</sup> (whether the radionuclide is exogenous or endogenous), which corresponds to the standards of radiolabelling.<sup>20</sup> CR is a promising radiation that may be used as a radioactive optical imaging in medicine, especially as optical sensors become even more sensitive. Hence, upon careful design of the (chemiluminescent) donor / (fluorescent) acceptor couple, CRET may appear as a convenient approach to transfer a portion of the blue-centered CR light towards the NIR region of the spectrum where tissues are more transparent. Hence, it appears necessary to better comprehend CR and develop CRET molecular probes for CLI.

## Notes and references

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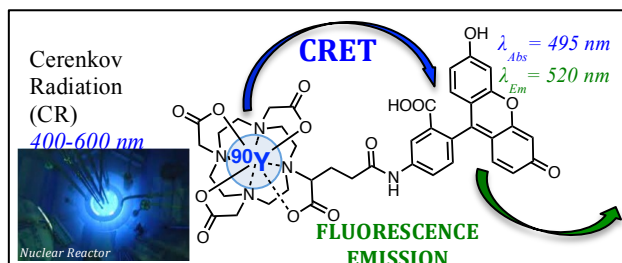
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