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Design and development of high bioluminescent resonance energy transfer efficiency hybrid-imaging constructs

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ABSTRACT

Here we describe the design and construction of an imaging construct with high bioluminescent resonance energy transfer (BRET) efficiency that is composed of multiple quantum dots (QDs; $\lambda_{em} = 655$ nm) self-assembled onto a bioluminescent protein, *Renilla* luciferase (Rluc). This is facilitated by the streptavidin–biotin interaction, allowing the facile formation of a hybrid-imaging construct (HIC) comprising up to six QDs (acceptor) grafted onto a light-emitting Rluc (donor) core. The resulting assembly of multiple acceptors surrounding a donor permits this construct to exhibit high resonance energy transfer efficiency (~64.8%). The HIC was characterized using fluorescence excitation anisotropy measurements and high-resolution transmission electron microscopy. To demonstrate the application of our construct, a generation-5 (G5) polyamidoamine dendrimer (PAMAM) nanocarrier was loaded with our HIC for in vitro and in vivo imaging. We envision that this design of multiple acceptors and bioluminescent donor will lead to the development of new BRET-based systems useful in sensing, imaging, and other bioanalytical applications.

Extensive effort has been invested in the design, development, characterization, and application of efficient light-emitting constructs using energy transfer [1]. Such optically active constructs have a strong impact on many scientific fields such as in the development of sensitive and selective biosensors [2–6], molecular medicine imaging [7–10], and others [1,6,7,11–16]. Part of this motivation for advanced resonance energy transfer (RET) platforms arises from the difficulty in tuning self-illuminating imaging constructs such as bioluminescent proteins for red-shifted emission. Although the Gambhir laboratory has made important advances in this regard [17], the limited (66-nm) bandwidth of these *Renilla* luciferase mutants combined with their intrinsically broad emission leaves little opportunity for multiplexing. However, the increased complexity of fabrication for RET systems naturally dictates the need for facile, rapid, and reproducible synthesis to truly revolutionize the application of these constructs. This can be achieved by developing methodologies in which individual reagent components are modified in such a way as to support self-assembly [18] of the desired light-emitting constructs. These light-emitting constructs often use fluorescent resonance energy transfer (FRET) [3,19] or bioluminescent resonance energy transfer (BRET) [4,20–23] mechanisms.

The optimization of a resonance energy transfer system involves proper matching of donor-to-acceptor energy levels in order to exhibit the desired emission characteristics. For example, to obtain high quantum yield and a high signal-to-noise ratio of the emitted energy, a large molar extinction coefficient, narrow emission, and good photostability from the acceptor are desired. Many commercially available organic dyes fulfill some of these requirements; however, they can be either sensitive to photobleaching or unstable under different pH values, exhibit small Stokes shifts, have narrow excitation and broad emission peaks, or have small molar extinction coefficients. Recent developments in the synthesis of colloidal

Abbreviations used: RET, resonance energy transfer; FRET, fluorescent resonance energy transfer; BRET, bioluminescent resonance energy transfer; QD, quantum dot; UV–Vis, ultraviolet–visible; CL, chemiluminescent; Rluc, Renilla luciferase; IVIS, In Vitro Imaging System; HIC, hybrid-imaging construct; QDSV, streptavidin-modified QDs; bRluc, biotin-modified Rluc; biotin-NHS ester, biotin-N-hydroxysuccinimide ester; G5-PAMAM, generation-5 polyamidoamine dendrimer; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; HR–TEM, high-resolution transmission electron microscopy; HIC-D, HIC encapsulated with G5-PAMAM dendrimer; PDD, peptide G5-PAMAM dendrimer; PBS, phosphate-buffered saline.

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semiconducting nanoparticles have expanded their use in various optically demanding applications such as FRET, BRET, and chemiluminescent resonance energy transfer (CRET) [20]. These luminescent quantum dots (QDs) have good photostability and high fluorescence quantum yield. Currently, commercially available QDs such as the widely used CdSe/ZnS and CdSe/CdTe, or the biocompatible InP/ZnS dots, can cover the full optical absorption spectrum while maintaining a narrow emission band (~45–50 nm) for application in sensing, imaging, and energy production (dye-sensitized solar cells or solar concentrators) [24]. Their broad ultraviolet–visible (UV–Vis) excitation band allows simultaneous excitation of multiple color QDs, allowing them to be considered as universal resonant energy donors. At the same time, their narrow emission allows for highly selective energy transfer to the appropriate fluorophore. However, the application of QD-based, UV–Vis FRET systems is limited in biological medium (tissues, organs, or cells) due to high biological attenuation of these wavelengths [15,21,22,25], which results in weak and diffuse emission. In addition, the use of high-power sources for QD excitation is required in order to penetrate the tissue. Consequently, high-energy excitation results in photobleaching of the acceptor dye and significant background from the biological media (autofluorescence). All of these factors are detrimental to the observed signal-to-noise (S/N) ratio. In addition, the broad emission of most fluorophores restricts the degree of multiplexing available for sensing or imaging constructs [26]. Although the broad UV–Vis absorption band of QDs makes them suitable as energy donors, their application as energy acceptors remained relatively limited until recently [4,27] and is mainly limited to sensing applications [28,29]. Du and coworkers [20] reported the design of QD-decorated chemiluminescent (CL) nanocapsules employing vinyl-encapsulated horseradish peroxidase conjugated to QDs. CL was generated using hydrogen peroxide and *p*-iodophenol as substrates. However, in vivo imaging using this construct was not shown, possibly due to the weak CL signal and complexity of the system. Here we describe a construct using QDs as the energy acceptor from a bioluminescence-based excitation energy source [21,23] placed in close proximity to the QDs.

Renilla luciferase (Rluc) is a bioluminescent protein that generates light as a result of a chemical reaction with its “luciferin” substrate, coelenterazine. This radiative energy can be used for direct imaging or as the excitation source for organic dyes or QDs. The emission spectrum of coelenteramide overlaps with the QD absorption spectra to provide resonance energy transfer that results in near-infrared QD emission for efficient biological matrix penetration (mm–cm) [7] and higher optical imaging sensitivity. A variation of this concept was previously demonstrated using self-illuminating QDs [21]. In this work, multiple Rluc (donors) were linked to a single QD (acceptor) through a zero-length cross-linker. This multiple donor–single acceptor method using a short Rluc–QD separation distance resulted in 56% BRET efficiency. The optimized construct was used for bioimaging using the In Vivo Imaging System (IVIS). This novel design allowed tissue imaging without the need for an exogenous excitation source. However, this application of multiple donors around a single acceptor required time-consuming conjugation chemistry.

In the current work, a new design is presented for a hybrid-imaging construct (HIC) based on the BRET principle that demonstrates high efficiency, self-assembly, and near-infrared illumination. The design is based on facile streptavidin–biotin chemistry, which enables a multiple acceptor–donor construct for superior BRET-based imaging. The HIC incorporates a network of streptavidin-modified QDs (QDSV) surrounding a biotin-modified Rluc (bRluc). A multiple acceptor–single donor configuration has not been demonstrated previously in a BRET study. Our system capitalizes on the fact that an increase in FRET efficiency was observed when multiple energy acceptors surrounded a single energy donor in several studies [3,5,6,30–32]. Due to the high quantum yield, large molar extinction coefficient, and broad absorption band [33] of the QD acceptors, a well-resolved, red-shifted, and stable emission is obtained. Bioluminescence emission from the donor (bRluc) in combination with the high excitation efficiency of the multiple QD acceptors results in low signal scattering and superior image quality for in vitro and in vivo imaging applications. The major advancement of this work comprises the ability to assemble the complete HIC using a facile technique that does not require complicated conjugation while exceeding the BRET efficiency of similar platforms that require significantly more synthetic manipulation. In addition, this is the first report of a BRET-based single donor–multiple acceptor construct, and this design was found to exceed the BRET efficiency observed in previously reported work.

Materials and methods

Materials

All purchased chemicals were used without further purification. Streptavidin-modified QDs emitting at 655 nm (QDSV) were purchased from Invitrogen (Carlsbad, CA, USA). Biotin-*N*-hydroxysuccinimide ester (biotin-NHS ester, 98% HPLC purified) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), ethylenediamine core generation-5 polyamidoamine dendrimer (G5-PAMAM), and fetal bovine serum (FBS) were purchased from Sigma–Aldrich. Targeted peptide (AKXVAAWTLKAAAZC)–G5 dendrimer conjugate was purchased from 21st Century Biochemicals. 96-Well microtiter plates with nonbinding surface were purchased from Corning (Corning, NY, USA). Microcon YM-100 (100 kDa MWCO) spin columns were purchased from Millipore (Billerica, MA, USA). Native coelenterazine was obtained from Prolume (Pinetop, AZ, USA). Zeba desalting spin columns (7 kDa MWCO), monobasic sodium phosphate anhydrous, and dibasic sodium phosphate heptahydrate were purchased from Thermo Fischer Scientific (Rockford, IL, USA). Dulbecco's modified Eagle's medium/Ham's F12 50:50 mix, 1% antibiotic–antimycotic solution, and trypsin–EDTA (ethylenediaminetetraacetic acid) were purchased from Cellgro (Manassas, VA, USA).

Renilla luciferase–biotin conjugation

Rluc was expressed and purified using methods developed in our laboratory [4]. Biotin-NHS ester was used to conjugate biotin to the free amine groups on Rluc. In a typical reaction, 1.1 nmol of Rluc was reacted with 11 nmol of biotin-NHS ester in 200 μl of 100 mM phosphate buffer (pH 8.0). The reaction was allowed to continue for 15 min prior to buffer exchange through Zeba desalting spin columns (7 kDa MWCO). The bRluc conjugates were buffer exchanged to 100 mM phosphate buffer (pH 7.0) and kept at 4 °C until further use. The bioluminescence emission of bRluc was measured and compared with Rluc, showing no significant effect of conjugation on the bioluminescence of Rluc.

Bioluminescence measurements

To synthesize self-assembled HIC, 1 pmol of bRluc was added to 100 mM phosphate buffer (pH 7.0) in a 96-well microtiter plate, followed by the incremental addition of QDSV from a 2-μM stock solution. This mixture was incubated for 15 min at 25 °C. To the final mixture volume of 150 μl, 50 μl of a 2-μg/ml solution of coelenterazine was injected, and luminescence was recorded using 486 ± 10 and 680 ± 10 nm emission filters with bioluminescent