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Developing a circularly permuted variant of *Renilla* luciferase as a bioluminescent sensor for measuring Caspase-9 activity in the cell-free and cell-based systems

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ABSTRACT

Biosensors and whole cell biosensors consisting of biological molecules and living cells can sense a special stimulus on a living system and convert it to a measurable signal. A major group of them are the bioluminescent sensors derived from luciferases. This type of biosensors has a broad application in molecular biology and imaging systems. In this project, a luciferase-based biosensor for detecting and measuring caspase-9 activity is designed and constructed using the circular permutation strategy. The spectroscopic method results reveal changes in the biosensor structure. Additionally, its activity is examined in a cell-free coupled assay system. Afterward, the biosensor is utilized for measuring the cellular caspase-9 activity upon apoptosis induction in a cancer cell line. In following the gene of biosensor is sub-cloned into a eukaryotic vector and transfected to HEK293T cell line and then its activity is measured upon apoptosis induction in the presence and absence of a caspase-9 inhibitor. The obtained results show that the designed biosensor detects the caspase-9 activity in the cell-free and cell-based systems.

1. Introduction

In multicellular organisms, apoptosis has critical roles in tissue hemostasis, development of embryo and metamorphosis of amphibians. This process is seen in two ways: intrinsic and extrinsic pathways which reinforce each other. In both pathways, the protease family members known as caspases are involved [1]. Caspases are cysteine proteases that cleave their targets after aspartate residues in the specific motifs [2,3]. Caspase cascade in both pathways begins with effector caspases: caspase-8 in the extrinsic pathway and caspase-9 in the intrinsic pathway. The effector caspases activate executive caspases in the downstream which is caspase-3/7. This cascade finally results in cell death occurrence [4]. Caspase-9 which is classified as an executioner caspase is expressed in a wide range of tissues in the form of a single-chain zymogen as procaspase-9. During apoptosis, caspase-9 activation is mediated by apoptotic protease activating factor-1 (APAF-1) and its active form, a homodimer composed of two small and two large subunits, is created [5,6]. Following the oligomerization and activation, caspase-9 cleaves its substrates by recognizing LEHD as a preferred sequence [3]. Furthermore, there exist pieces of evidence that highlight the critical role of caspase-9 activity not only in apoptosis but also in differentiation progress [7,8]. Regarding this pivotal importance of caspase-9 in the cellular phenomenon, acquisition of approaches to detect caspase-9 activity especially in real time is invaluable.

Today, protein engineering has become a powerful tool which has paved the way for manipulation of proteins to achieve the novel ones with different and desired behavior. One of the creative and efficient approaches in protein engineering is the circular permutation. Circularly permutated protein is a form of a protein in which

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the original N and the C terminus of a protein covalently bind to each other and the novel terminus is created at the different positions. Notably, permutation event is an evolutionary feature in several proteins such as Concanavalin A from *Canavalia ensiformis* [9,10]. This feature can be used to manufacture the engineered protein to study its biological activity. Usually, in the genetic engineering level, the original N and C terminus covalently link to a linker sequence, such that the cleavage of that linker helps the protein to gain its functional structure again [11]. This approach has been applied to numerous enzymes to create engineered enzymes, with the purpose of controlling enzymatic activity, development of chemotherapeutic agents, and detection of the proteases activity. Amongst the various engineered enzymes, ribonuclease A and firefly luciferase can be mentioned which have been circularly permutated [12–14]. Luciferase-aided probing system has also been applied to investigate the molecular dynamics of proteins [15].

Herein, considering the importance of caspase-9 activity in some cellular processes and lack of a cell-based biosensor for measuring its activity, we took advantage of circular permutation to design and develop a luciferase-based biosensor to detect cellular caspase-9 activity.

Nowadays, luciferase enzymes are widely applied in the study and revealing of the biological processes. The luciferases which are widely used in biomedical research are the *firefly* luciferase (Fluc), the *Renilla* luciferase (Rluc); and the *Gaussia* luciferase (Gluc) [16]. Recently, a variant of Rluc containing eight point mutations, so-called Red-shifted *Renilla* luciferase variant (RLuc8), has been developed. It has been reported that RLuc8 has enhanced stability in murine serum and higher light emission than the native RLuc [17,18]. Considering the advantages of RLuc8 for *in vivo* imaging and our future projects, we profit from this variant to construct the biosensor. The biosensor has been built by splitting luciferase into two fragments and connecting them through a protease cleavage site (LEHD). It is anticipated that these structural changes would result in inactivation of the enzyme, which is later confirmed by structural and functional studies. However, this inactive form of the enzyme is expected to retrieve its activity in the presence of active caspase-9 and the occurrence of proteolytic cleavage. Therefore, the spliced luciferase regains its bioluminescent activity and emits light in the presence of substrate. In this coupled assay system, the intensity of emitted light is attributed to the caspase-9 activity. This assay system could be applicable as a cell-free system for measuring the exogenous and endogenous caspase-9 activity, and a cell-based system for detecting the cellular caspase-9 activity. Therefore, the newly designed biosensor, CP-RLuc8, enables us the study of exogenous and endogenous caspase-9 throughout different cellular conditions.

2. Materials and methods

2.1. Design of circularly permuted form of RLuc8 and in silico studies

In order to design the circularly permuted form of RLuc8 (CP-Rluc8), the peptide bond between residues 229 and 230, in the protein sequence of RLuc8 (accession number: ABO28825.1), was considered as the splicing site. The small and large fragments flanked to this site were replaced with each other and a linker sequence, LEHDG, was set in the middle. Later, amino acid sequences of two proteins, RLuc8 and CP-RLuc8 were submitted as an input for I-TASSEER Server [19–21]. The obtained models, using this server, were visualized by SPDBV_4.10_PC and the structural changes were evaluated.

2.2. Prokaryotic plasmid construction

RLUC8 gene was synthesized by Generay Company. Splicing by Overlap Extension PCR (SOE-PCR) method was applied to create and amplify the CP-RLuc8. Initially, the small fragment of 688–933 bp and the large fragment of 1–687 bp were created using two pairs of primers, cloning and linker primers, by an ordinary PCR. The large and small fragments were amplified and modified in order to have a unique sequence of a linker, LEHDG. Large fragment was amplified by R-linker primer (5′-CTGGAGCATGACGGTGCTTC-CAAGGTG -3′) and a unique enzyme site of BamHI using the F-cloning primer (5′-ATGGGATCCAAGCCCGACGTCGTC-3′). The small fragment was amplified by F-linker primer (5′-CTGGAGCAT-GACGGTGCTTCCAAGGTG-3′) and a unique enzyme site of XhoI using R-cloning primer (5′-ATGCTCGAGGCCTCCCTTAACG -3′). In the second step, the amplified small and large fragments were used as a template for SOE-PCR, using F and R-Cloning primers, to produce and amplify the rearranged RLuc8 gene. SOE-PCR was performed by Pfu polymerase in a two-step reaction. The first step was 10 cycles, 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min. In the second step F and R-cloning primers added to the tube and PCR continued identical to the first step PCR.

The amplified CP-RLuc8 was digested using BamHI and XhoI and ligated into the digested expression vector pET-28a. The ligation product then was transformed into the replicative bacterial host DH5α. Positive colonies were selected using colony-PCR. In order to further confirm the successful cloning of CP-RLuc8 gene, the recombinant vector was extracted, single and double digestion of recombinant vector were performed, and cloning was confirmed by sequencing analysis.

2.3. Eukaryotic plasmid construction

To study the CP-RLuc8 activity in the eukaryotic cells, it must be sub-cloned into a eukaryotic expression plasmid. Therefore, the designed CP-RLuc8 was amplified using a new set of primers that introduced Kozak consensus sequence to the CP-RLuc8 gene to have a proper translation. The forward primer 5′-ATA CCA CAA CCA TGG GAA AGC CCG ACG-3′ and reverse primer 5′-ATT GCG GCC GCT TAG CCT CCC TTA ACG-3′ respectively contained unique restriction sites for Bstx-I and Not-I enzymes. The newly synthesized gene was digested with the aforementioned enzymes and ligated into the similarly digested eukaryotic expression plasmid pIRES2-EGFP.

2.4. Expression and purification of recombinant proteins: RLuc8, CP-RLuc8, and human caspase-9

To overexpress and purify recombinant proteins in *E. coli* system, recombinant plasmids were transformed into the expression host BL21 (DE3). Expression of RLuc8 and CP-RLuc8 was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h at 32 °C. Purification of RLuc8 and CP-RLuc8 containing histidine-tag was performed on Ni-NTA agarose column (QIAGEN).

Dialysis of eluted fractions of purified protein, containing imidazole, was done against 2 L of 100 mM phosphate buffer pH 7.0 for 24 h, to omit the interference of imidazole with luciferase activity. The glycerol stocks of purified CP-RLuc8 were prepared and stored at −20 °C.

Vector pET23b-Casp9-His (Addgene) was transformed into BL21 (DE3). Overexpression and purification steps for caspase-9 was followed as described in Roschitzki-Voser et al. paper [22].

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3. Structural analysis of CP-RLuc8 using fluorescence and circular dichroism (CD) spectroscopy

3.1. Fluorescence spectroscopy

Fluorescence spectra were registered on spectrofluorometer Cary Eclipse with excitation at 280 and 295 nm. Cell path length was 1 cm and band width of excitation and emission monochromators were 5 nm. The spectra were recorded at room temperature in phosphate buffer saline (PBS), pH 7.2. The protein concentration was 0.01 mg/ml for intrinsic fluorescence measurement.

3.2. Circular dichroism spectroscopy

Circular dichroism spectra were recorded on Aviv Model 215 circular dichroism spectrometer at room temperature using 1 mm path length cuvette. For obtaining the circular dichroism spectra in far-UV region the protein concentration was 0.14 mg/ml. The mean residue molar ellipticity (MRW) in deg. cm^2. $dmol^{-1}$ was calculated using the following equation:

$$[\theta] = \frac{\theta \times 100\ \mathbf{MRW}}{\boldsymbol{cl}}$$

Where c is the concentration in mg/ml, l is the cell path length in centimeter, MRW is mean residue weight of the protein, and θ is the measured ellipticity in degree at wavelength λ [23,24].

4. Functional analysis of CP-RLuc8

4.1. Recombinant human caspase-9 activity assay using colorimetric kit

Recombinant human caspase-9 activity was assayed by LEHD-ρNA, as a chromogenic substrate, in a 2X reaction buffer containing 25 mM HEPES, pH 7.5, 0.1% CHAPS, 5% (v/v) sucrose, 5 mM DTT, 2 mM EDTA, under the standard protocol.

4.2. Recombinant human caspase-9 activity assay using CP-RLuc8 in vitro

This assay is a 2-step coupled reaction. In order to assess the CP-RLuc8 activity, caspase-9 activity is required. The first step of the assay is activating CP-RLuc8 (0.018 mg/ml) in the presence of caspase-9 (0.018 mg/ml), 2X reaction buffer, and the enzyme substrate which is 0.5 μg/ml coelentrazine in ethanol 0.8% and 90 s incubation at room temperature is required. In order to examine the effect of circular permutation on luciferase activity, a reaction containing the intact RLuc8 (0.018 mg/ml) and a reaction containing CP-RLuc8 without caspase-9 were assayed as well. The detection limit of probe was also evaluated by varying the concentration of purified caspase-9 (from 0.001 to 0.025 mg/ml) against CP-RLuc8 (0.018 mg/ml). All the assays were performed using a bioluminescent system (Berthold Technologies).

4.3. Cell culture, apoptosis induction and endogenous caspase-9 activity measurement using purified CP-RLuc8

The MCF-7 cell line (ATTC) was cultured in medium containing RPMI (Gibco) and 10% FBS and 1% penicillin/streptomycin (Gibco). The cells were maintained at 37 °C in an incubator with 5% CO2. In order to induce apoptosis, cells were treated with doxorubicin, 5 μM (Ebendoxo, EBEWE Pharma Ges). Sampling was done at 6, 12, and 24 h after apoptosis induction. Cell pellet was stored at −80 °C for the later analysis.

To prepare cell lysate, Cell Culture Lysis Reagent (CCLR) containing 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, and 10% (v/v) glycerol were used. Harvested cells of different experiments at predetermined intervals were mixed with 100 μl of CCLR buffer and the resulting cell lysates were stored at −80 °C. Protein concentration determination of cell lysates was done by standard Bradford method.

Cell lysate prepared by CCLR at predetermined intervals with equal amounts of total protein were subjected to endogenous caspase-9 activity measurement using purified CP-RLuc8. Each test tube containing cell lysate suspended in 2X reaction buffer and CP-RLUC8. After 90 s incubation at room temperature, coelenterazine 0.5 μg/ml was added to the cocktail. Light emission was measured using a bioluminescent system.

4.4. Transient transfection of HEK293T cells with pIRES2-CP-RLuc8, apoptosis induction and cellular caspase-9 activity measurement

Since HEK293T cells are more suitable for transient transfection, the pIRES2-CP-RLuc8 was transfected to this cell line using Lipofectamine 2000 transfection reagent (Invitrogen) according to the standard protocol. To induce apoptosis, the transfected cells were treated with doxorubicin 5 μM (Ebendoxo, EBEWE Pharma Ges), after 48 h. Sampling was done 6 h after apoptosis induction. The transfected cells without treatment were set as the negative control. Cell pellet was stored at −80 °C for the later analysis.

Cell lysates containing CP-RLuc8 with equal amounts of total proteins were subjected to endogenous caspase-9 activity measurement. Coelenterazine (0.5 μg/ml) was added to each test tube containing cell lysate suspended in 2X reaction buffer. The light emission was measured instantly using a bioluminescent system.

In addition, to examine whether CP-RLuc8 is specifically cleaved by caspase-9, a specific caspase-9 inhibitor, Ac-LEHD-CMK (10 μM) (Santa Cruz), was added to the cell lysates. Then they were subjected to endogenous caspase-9 activity measurement.

4.5. Hoechst/PI staining to detect the cell death

To detect apoptosis using a microscopic method, the cells were double stained with fluorescent dyes, propidium iodide (PI) and Hoechst 33342. The staining solution was added to the cells at a final concentration of 0.5 μg/ml. The cells were incubated at 37 °C for 15 min, subsequently the samples were visualized by fluorescent microscope (Optika).

4.6. Real time imaging of apoptotic cells

In order to visualize the intact apoptotic cells, the HEK293T cells were transfected with plasmid containing CP-RLuc8. Two days after transfection, the transfected cells were detached and seeded into a 96-well plate with different cell number (from μ000 to 10000 cells/well). The cells were treated with the apoptotic drug and incubated. 6 h after incubation the plate was placed into the imaging system (Bio-Rad ChemiDoc touch imaging system), coelenterazine was added and then imaging was done.

4.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. One-way and two-way Analysis of Variance (ANOVA), followed by Dunnett's post-hoc test, when appropriate, were used to determine significant differences. P values < 0.05 were considered significant. All experiments were performed in three independent replicates and the mean is reported with the corresponding standard deviations.

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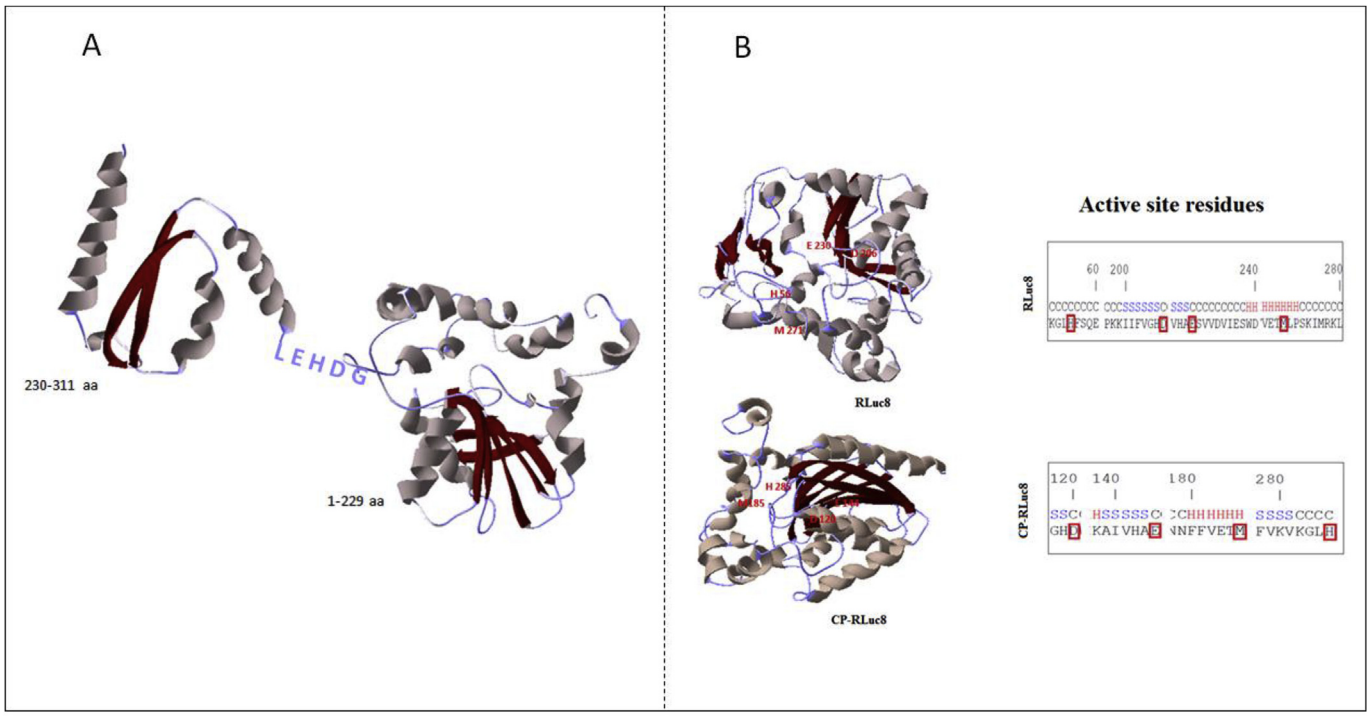

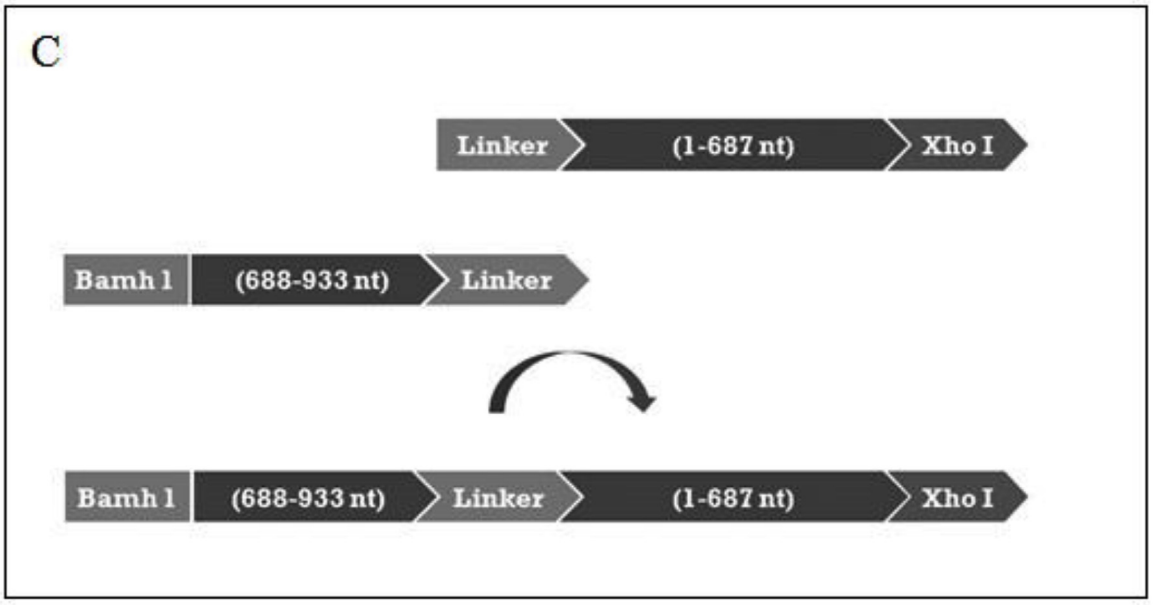

Fig. 1. Design and prediction of CP-RLuc8 structural changes. A) Schematic representation of CP-RLuc8 in which N and C domains of RLuc8 are fused to the linker, LEHDG, in reverse orientation. B) Models of Rluc8 and CP-RLuc8 are depicted by SPDBV_4.10_PC based on predicted structures by I-TASSER server. The position of active site residues in RLuc8 and CP-RLuc8 have been shown on the right hand of each model. C) A schematic representation of genetic engineering of RLuc8 to CP-RLuc8. Large and small fragments, respectively 1–687 bp and 688–933 bp were amplified using ordinary PCR and then the complete rearranged fragment containing linker were amplified using SOE-PCR.

5. Results and discussion

5.1. CP-RLuc8 was designed, constructed and purified

In the current project, the strategy to construct the biosensor is the circular permutation. In this design, RLuc8 is split into two fragments (N and C domains), their original positions swapped, and then two fragments are connected with a linker. In this construct, the linker is LEHDG in which LEHD is the target sequence of caspase-9 and G has been placed to maintain the structural flexibility. Since the biosensor's construct carries the target sequence of caspase-9, it is expected that in the presence of caspase-9 the construct is cleaved. Following its cleavage, luciferase would fold into its true structure and regain its bioluminescent activity which is detectable by a luminometer.

Here the major challenge in the design of a functional circularly

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permuted structure was choosing a precise cleavage site and the His-tag position in protein sequence, such that enzyme regains its activity despite splitting and also having a 6-histidine sequence in the middle of it. Owing this complexity, the split site of RLuc8 was selected based on a previous report which showed split Renilla luciferase worked well if the cleavage site was placed between the residues of 229 and 230 [25]. To determine the position of His-tag at N-terminus, C-terminus or both termini, *in silico* study was performed using I-TASSER server [19–21]. Using this method, the best model with minimum changes in protein structure, especially at active site, could be predicted and chosen. Regarding the permutation in RLuc8, rearranged protein sequence with different states of His-tag at N-terminus, C-terminus and both termini were designed. As illustrated in Fig. 1A, in this design the LEHDG sequence is flanked with a small fragment at N-terminus, 230–311 residues, and a large fragment at C-terminus, 1–229 residues. Designed rearranged sequences of proteins were submitted to the I-TASSER server. Comparison of the obtained models showed that locating His-tag at N, C or both termini yields the similar results. The model also reveals that the active site conformation of CP-RLuc8 is the same as RLuc8, therefore, it is expected the catalytic triad of CP-Rluc8 could maintain the enzymatic activity. However, this triad in RLuc8 has been reported as D120, E144, and H285 [26], but in CP-RLuc8 is located at H56, D206, and E230 residues (Fig. 1B).

To construct the CP-RLuc8 gene (Fig. 1C), as elaborated in experimental section, two small and large fragments, 688–933 bp and 1–687 bp, were separately amplified using PCR, afterwards the complete 942 bp sequence was created using SOE-PCR.

RLuc8 (Fig. 1S.A) and CP-RLuc8 (Fig. 1S.B) were expressed and purified. Also since caspase-9 is required for CP-RLuc8 activation, all the expression and purification steps were performed to produce this enzyme (Fig. 1S.C) and its activity was examined using a colorimetric kit. Result shows that it is active and applicable for the next steps (Fig. 1S.D).

5.2. Circular permutation caused structural changes in RLuc8

It was expected that manipulating RLuc8 sequence by circular permutation, accompanies with some structural changes, therefore, the structure of the intact luciferase (RLuc8) and its circularly permuted variant (CP-RLuc8) were analyzed using fluorescence spectroscopy and circular dichroism. In Fig. 2S.A, the intrinsic fluorescence spectra of RLuc8 and CP-RLuc8 show a strong reduction in the fluorescence intensity of CP-RLuc8. This result indicates that the structure of CP-RLuc8 becomes less compact due to formation of the circular permuted form [27]. Circular dichroism spectra of RLuc8 and CP-RLuc8 also reveal different percentages of regular secondary structure in two variants (Fig. 2S.B) as evidenced by negative signal between 210 and 240 nm. It seems manipulation of RLuc8 sequence to make the circular permuted form leads to decreasing intensity of the negative signal at 210–240 nm and appearance of an intense negative signal at about 198 nm, which implies a transition from the regular secondary structures to random coil [24]. These evidences reveal the structural changes upon circular permutation of luciferase.

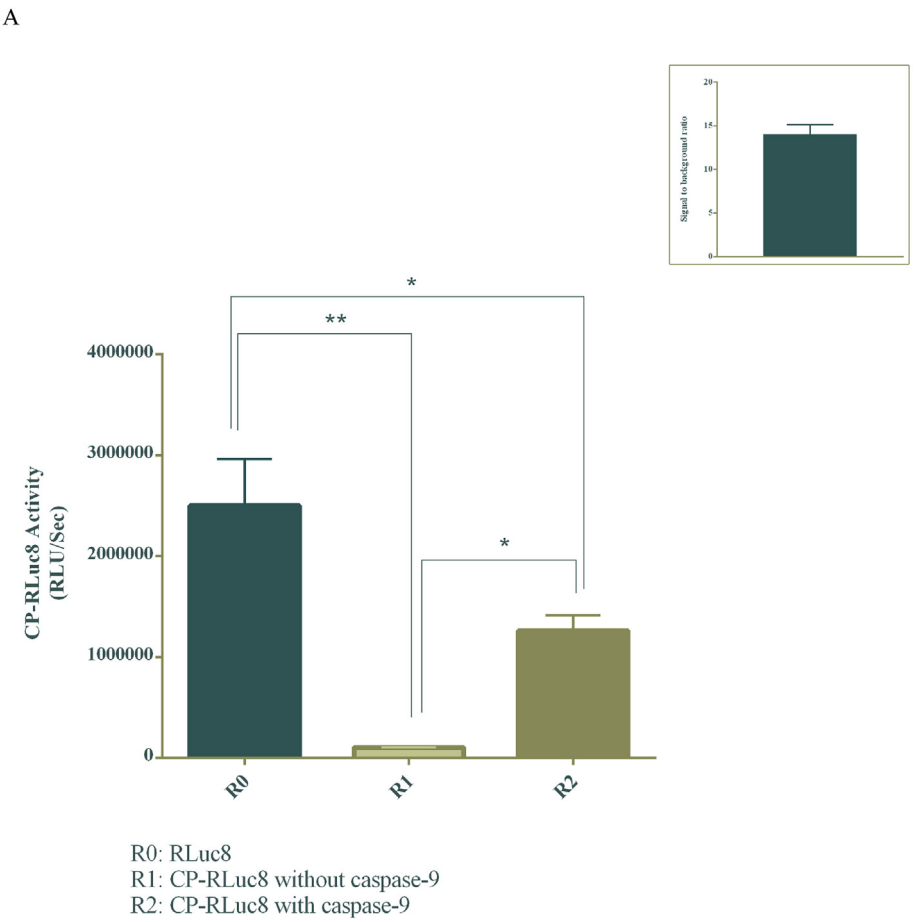

Fig. 2. Luciferase activity measurement. A) The assay was done for RLuc8 (R0), CP-RLuc8 in the absence (R1), and presence of caspase-9 (R2). The inset shows the signal to background intensity of CP-Rluc8 activity. Two-way ANOVA followed by Dunnett's post-hoc test was performed to analyze the data. P values < 0.05 were considered significant, as indicated by the asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

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5.3. CP-RLuc8 detects caspase-9 activity in a coupled assay system

To follow the effect of circular permutation on CP-RLuc8 activity and measure the activity of its purified form after cleavage, the purified RLuc8 and CP-RLuc8 were subjected to a coupled assay in 2X reaction buffer and in the presence of coelentrazine. The results (Fig. 2A) show the bioluminescent activity of intact luciferase (RLuc8) as 2.5×10^6 Rlu/Sec. The bioluminescent emission of CP-RLuc8 in the absence of caspase-9 is 0.1×10^6 Rlu/Sec while in the presence of caspase-9 the intensity of light emission increases nearly 12 folds (1.2×10^6 Rlu/Sec). The observed emission for non-cleaved CP-RLuc8 was not expected, which is possibly due to the base activity of circularly permuted enzyme, and the obtained signal to background ratio (Fig. 2, inset) is a reasonable ratio. Searching for other split sites can reveal more optimal split sites with higher ratio of signal to background. The comparison of emitted light for intact luciferase, CP-RLuc8 and cleaved CP-RLuc8 reveals that approximately 96% of the luciferase activity is lost during circular permutation, and proteolytic cleavage of CP-RLuc8 restores 50% of the activity. It is noteworthy that since the reaction is a coupled assay made up of two enzymes in which CP-RLuc8 is a substrate for caspase-9, it seems that the incubation time of two enzymes is an important point to be considered. Our experiences show that the enzyme cocktail should be incubated for 90–120 s. It could be the necessary time for interaction between two enzymes or having the opportunity to find an appropriate conformation. The detection limit for biosensor was identified by varying the concentration of purified caspase-9 (Fig. 3S). The study showed that CP-RLuc8 can detect 5 μg/ml of caspase-9. It can be concluded that to have an acceptable signal per 1 μg of the caspase-9, at least 3.5 μg of probe is needed.

5.4. CP-RLuc8 senses the endogenous caspase-9 activity during apoptosis

Caspase-9 is expected to detect CP-RLuc8 as the substrate and causes bioluminescent activity, similar to the cell-free systems. To examine the detection of CP-RLuc8 by caspase-9 during apoptosis, we induced apoptosis in MCF-7 cells. To accomplish this, MCF-7 cells were treated with doxorubicin hydrochloride and harvested at specific time points of 4, 12, and 24 h. The collected samples, normalized based on total protein, were subjected to the coupled assay. Results show the luciferase activity changes during apoptosis progress (Fig. 3). Such that the maximum caspase-9 activity is observed between 4 and 12 h after apoptosis induction and then gradually declines. To determine whether increased bioluminescence intensity actually correlated with caspase-9 activity, the caspase-9 activity in samples was also assayed using the colorimetric kit. The result of colorimetric kit matched with the result of assay by CP-RLuc8 (Fig. 4S).

Subsequently, to examine the ability of CP-RLuc8 to be used in a cell-based biosensor and detecting caspase-9 activity and apoptosis, the CP-RLuc8 gene was sub-cloned into the eukaryotic plasmid, pIRES2. The pIRES2-CP-RLuc8 was transfected into the HEK293T cell line and 48 h later, the transfected cells were exposed to doxorubicin as apoptosis inducer. In order to have an estimation

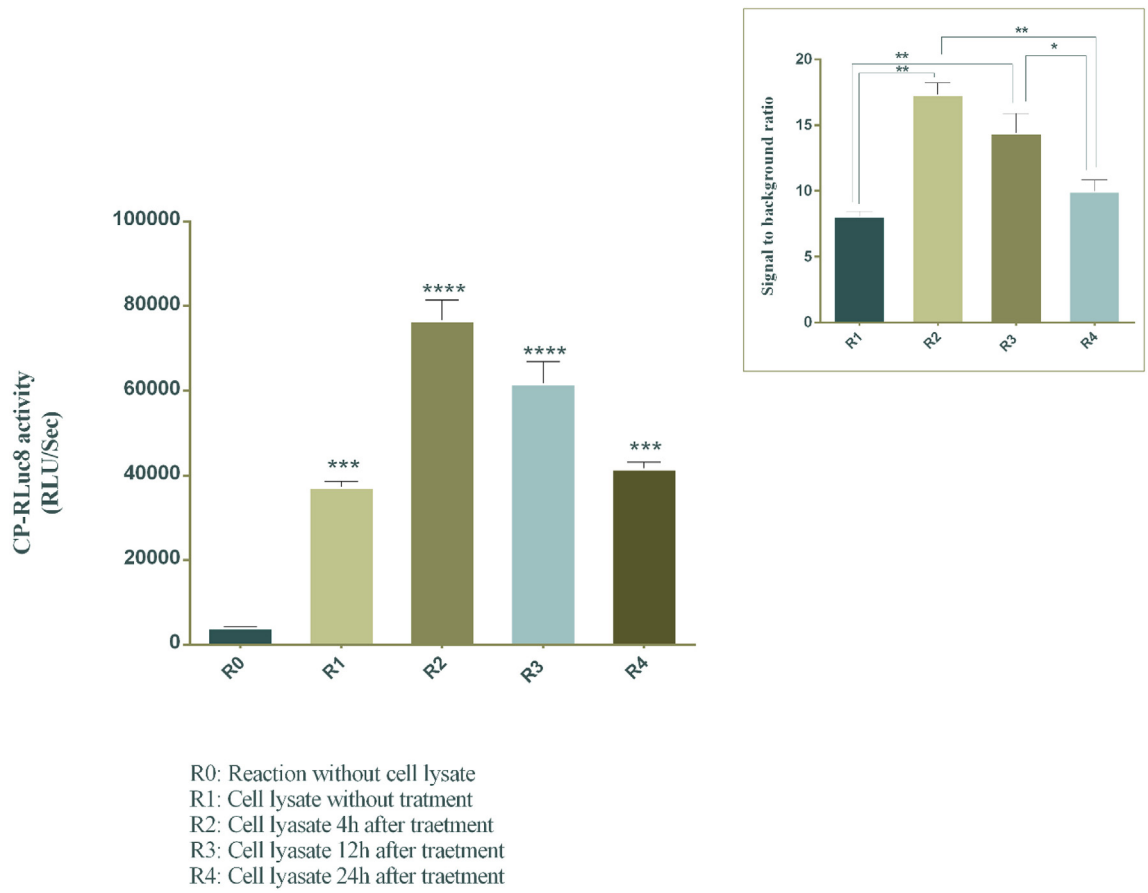

Fig. 3. Measurement of endogenous caspase-9 activity upon apoptosis induction using CP-RLuc8 biosensor. The caspase-9 activity was assayed at specific time intervals of 0, 4, 12, and 24 h after apoptosis induction. The inset shows the signal to background ratios of R1 to R4. The data are means ± S.D. (error bars) of triplicate experiments. One-way and two-way ANOVA followed by Dunnett's post-hoc test were performed to analyze the data. P values < 0.05 were considered significant, as indicated by the asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

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of cell death, Hoechst/PI staining was performed. As depicted in Fig. 4A, cells treated with doxorubicin show intensified red spots (indicated by arrows), which is an evidence of increased cell death compared to non-treated cells. In another experiment the bioluminescent signal of treated intact cells were also detected directly using an imaging system. As it is elucidated in Fig. 4B, the intensity of signals varies by changing the number of apoptotic cells. In parallel, the caspase-9 activity of treated and non-treated cells was measured by adding coelenterazine to the cell extracts, normalized based on total protein. On the other hand, as indicated in Fig. 4C, the non-treated cells containing CP-RLuc8 (R1) in contrast to the non-transfected cells (R0) show an insignificant luciferase activity that it can be due to the base activity of caspase-9 in the cells. Furthermore, the comparison of samples related to the treated and non-treated cells (R2 and R1, respectively) shows higher luciferase activity which implies increased caspase-9 activity during apoptosis induction.

To ensure the measured activity is related to caspase-9 and there is no cross-reactivity, we used a specific inhibitor against caspase-9. Hence, the CP-RLuc8 activity was examined again in the presence of Ac-LEHD-CMK (reactions; R0′, R1′ and R2′). As depicted in Fig. 4B, R0 and R0′ are similar and do not show any activity. In R1′, the small amount of detected base activity of caspase-9 decreases by adding the inhibitor. Eventually, in R2′, which is the reaction containing the treated cells, caspase-9 activity decreases to the amount of those samples without CP-RLuc8. From the results obtained, it can be concluded that the reported activity is due to the caspase-9 activity rather than cross-reactivity. Therefore, CP-RLuc8 biosensor can provide us with the ability for measuring the endogenous caspase-9 activity during cellular phenomena such as apoptosis. It is noteworthy since coelenterazine is a cell permeable compound; it can be used for intact cells such that biosensor acts as an internal sensor without adding any reagents.

6. Conclusion

Biosensors and whole cell biosensors are the valuable tools consisting of biological molecules and living cells. They can sense a special stimulus on a living system as a bio-recognition component [28]. Today, a plenty of cell-based biosensors have been reported which can be implemented for detecting various cellular responses to the different environmental changes, toxins, and pathogens [29–34]. Among them, the biosensors applicable in the detection of apoptosis are of particular importance, due to their application in drug discovery against disease like cancer. A good example of such system is a cell-based biosensor for detecting apoptosome formation upon apoptosis and differentiation induction [7,32]. Another interesting example is the caspase tracker system which is designed for detecting anastasis and non-apoptotic caspase activity *in vivo* [31]. The bioluminescent capsules are another example of developed systems for live cell imaging [35,36]. In this regard, circular permutation is a recent approach in protein engineering with the potential to design novel biosensors [11,15], and circularly permuted variants of the green and red fluorescent proteins have been broadly studied as the physiological indicators [37–40]. In a recent project, this strategy has been applied to develop biosensor for measuring caspase-3 activity using *firefly* luciferase in which DEVD, caspase-3/7 target sequence, is inserted into circularly permutated protein [13]. In the other report, GloSensor, caspase 3/7 biosensor using firefly luciferase has been developed to image caspase-3/7 activity in live cells and animal models. In this report, also DEVD is considered as a target sequence for caspase-3/7 activity [14].

In the present study, we have taken advantage of this strategy to design and develop a novel biosensor. The biosensor can be utilized

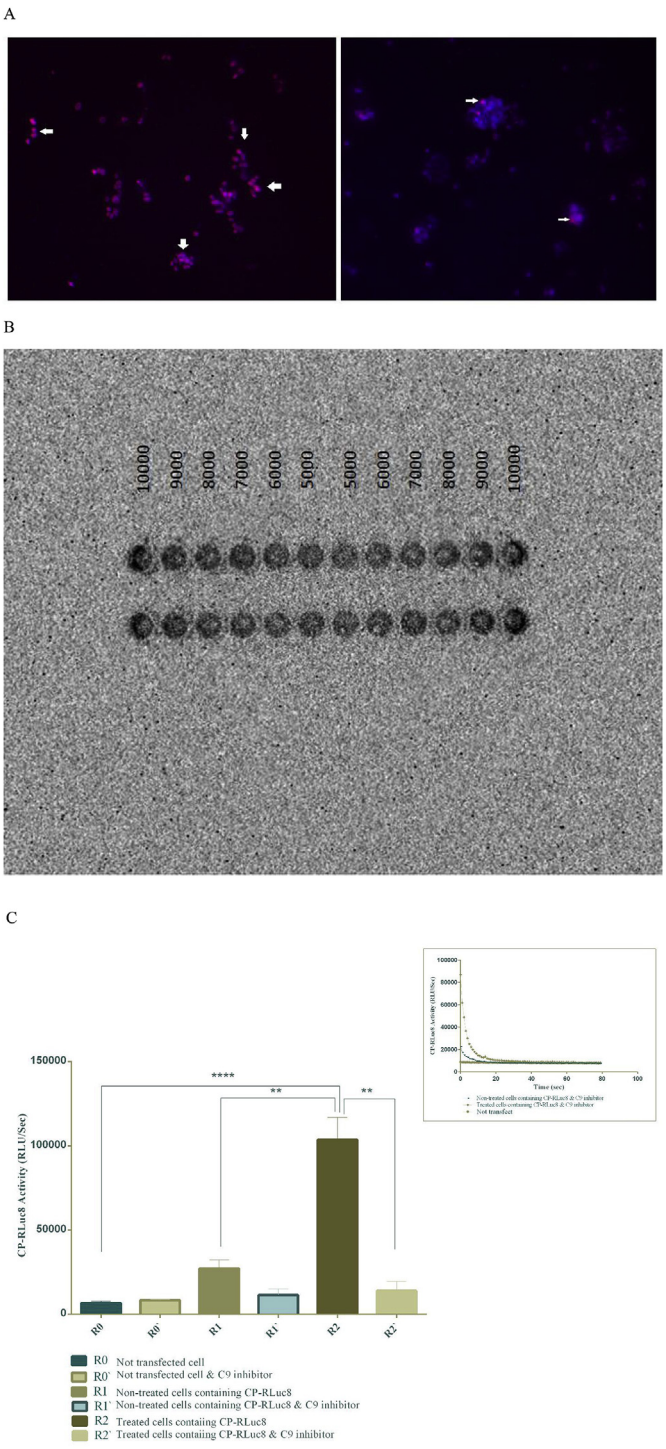

Fig. 4. Evaluation of A) the cell death 6 h after apoptosis induction using fluorescent microscopy (magnification 10X, the arrows show dead cells), B) real time imaging of apoptotic cells, and C) measurement of the endogenous caspase-9 activity 6 h upon apoptosis induction in transfected cells containing CP-RLuc8 biosensor. The caspase-9 activity was assayed for non-transfected cells in the absence and presence of caspase-9 inhibitor (R0 and R0′); transfected but non-treated cells in the absence and presence of caspase-9 inhibitor (R1 and R1′); transfected and treated cells in the absence and presence of caspase-9 inhibitor (R2 and R2′). The inset shows time course decrease of CP-RLuc8 activity in the presence of caspase-9 inhibitor. The data are means ± S.D. (error bars) of triplicate experiments. Two-way ANOVA followed by Dunnett's post-hoc test was performed to analyze the data. P values < 0.05 were considered significant, as indicated by the asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

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to study caspase-9 activity in a cell-free and also cell-based system. It is noteworthy that there is evidence which shows caspase-9 has a higher affinity for its physiological substrate, pro-caspase 3, rather than the synthetic substrate like LEHD-AFC [41]. However, in the current design luciferase become the caspase-9 substrate having an LEHD target site and it is expected to imitate physiological conditions better than the other synthetic substrates. The biosensor detects apoptosis based on caspase-9 activity, on the upstream of caspase-3/7 in caspase cascade which is suitable for identification of apoptosis at the steps earlier than caspase-3/7. As mentioned in previous studies, caspase-9 plays a pivotal role in differentiation and apoptosis regulation and execution [7,8,42]. Therefore, regarding to the central role of caspase-9 in critical cellular processes, the biosensor can provide us with more information on cellular and biochemical studies. Moreover, the introduced biosensor has the potential to be implemented as a whole cell biosensor for *in vitro* and *in vivo* imaging studies of apoptosis in future, especially in the studies related to the drug screening.

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Abbreviations

CP	Circular Permutation
Rluc	*Renilla* luciferase
Fluc	*Firefly* luciferase
Gluc	*Gaussia* luciferase
CP-RLuc8	Circularly Permuted *Renilla* luciferase8
PI	Propidium iodide

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.11.009.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.11.009.

References

[1] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (4) (2007) 495–516.
[2] D.W. Nicholson, Caspase structure, proteolytic substrates, and function during apoptotic cell death, Cell Death Differ. 6 (11) (1999) 1028–1042.
[3] G.P. McStay, G.S. Salvesen, D.R. Green, Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways, Cell Death Differ. 15 (2) (2008) 322–331.
[4] M. Brentnall, et al., Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis, BMC Cell Biol. 14 (2013) 32.
[5] C. Pop, et al., The apoptosome activates caspase-9 by dimerization, Mol. Cell 22 (2) (2006) 269–275.
[6] E.N. Shiozaki, J. Chai, Y. Shi, Oligomerization and activation of caspase-9, induced by Apaf-1 CARD, Proc. Natl. Acad. Sci. U. S. A. 99 (7) (2002) 4197–4202.
[7] S. Akbari-Birgani, et al., Delay in apoptosome formation attenuates apoptosis in mouse embryonic stem cell differentiation, J. Biol. Chem. 289 (24) (2014) 16905–16913.
[8] T.V. Murray, et al., A non-apoptotic role for caspase-9 in muscle differentiation, J. Cell Sci. 121 (Pt 22) (2008) 3786–3793.
[9] B.A. Cunningham, et al., Favin versus concanavalin A: circularly permuted amino acid sequences, Proc. Natl. Acad. Sci. U. S. A. 76 (7) (1979) 3218–3222.
[10] U. Heinemann, M. Hahn, Circular permutation of polypeptide chains: implications for protein folding and stability, Prog. Biophys. Mol. Biol. 64 (2–3) (1995) 121–143.
[11] Y. Yu, S. Lutz, Circular permutation: a different way to engineer enzyme structure and function, Trends Biotechnol. 29 (1) (2011) 18–25.
[12] P. Plainkum, et al., Creation of a zymogen, Nat. Struct. Biol. 10 (2) (2003) 115–119.
[13] R. Cheraghi, et al., Structural and functional effects of circular permutation on firefly luciferase: in vitro assay of caspase 3/7, Int. J. Biol. Macromol. 58 (2013) 336–342.
[14] S. Galban, et al., Imaging proteolytic activity in live cells and animal models, PloS One 8 (6) (2013) e66248.
[15] S.B. Kim, M. Sato, H. Tao, Circularly permutated bioluminescent probes for illuminating ligand-activated protein dynamics, Bioconjug. Chem. 19 (12) (2008) 2480–2486.
[16] C.E. Badr, B.A. Tannous, Bioluminescence imaging: progress and applications, Trends Biotechnol. 29 (12) (2011) 624–633.
[17] A.M. Loening, et al., Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output, Protein Eng. Des. Sel. 19 (9) (2006) 391–400.
[18] A.M. Loening, A.M. Wu, S.S. Gambhir, Red-shifted Renilla reniformis luciferase variants for imaging in living subjects, Nat. Methods 4 (8) (2007) 641–643.
[19] A. Roy, A. Kucukural, Y. Zhang, I-TASSER: a unified platform for automated protein structure and function prediction, Nat. Protoc. 5 (4) (2010) 725–738.
[20] J. Yang, et al., The I-TASSER Suite: protein structure and function prediction, Nat. Methods 12 (1) (2015) 7–8.
[21] Y. Zhang, I-TASSER server for protein 3D structure prediction, BMC Bioinf. 9 (2008) 40.
[22] H. Roschitzki-Voser, et al., Human caspases in vitro: expression, purification and kinetic characterization, Protein Expr. Purif. 84 (2) (2012) 236–246.
[23] S.M.a.P. Kelly, N.C, The use of circular dichroism in the investigation of protein structure and function, Curr. Protein Pept. Sci. 1 (2000) 349–384.
[24] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism, Biochim. Biophys. Acta 1751 (2) (2005) 119–139.
[25] S.B. Kim, et al., High-throughput sensing and noninvasive imaging of protein nuclear transport by using reconstitution of split Renilla luciferase, Proc. Natl. Acad. Sci. U. S. A. 101 (32) (2004) 11542–11547.
[26] J. Woo, M.H. Howell, A.G. von Arnim, Structure-function studies on the active site of the coelenterazine-dependent luciferase from Renilla, Protein Sci. 17 (4) (2008) 725–735.
[27] J.R. Lakowicz, Principles of frequency-domain fluorescence spectroscopy and applications to cell membranes, Subcell. Biochem. 13 (1988) 89–126.
[28] L. Bousse, Whole cell biosensors, Sensor. Actuator. B Chem. 34 (1–3) (1996) 270–275.
[29] P. Banerjee, S. Kintzios, B. Prabhakarpandian, Biotoxin detection using cell-based sensors, Toxins 5 (12) (2013) 2366–2383.
[30] E. Eltzov, et al., On-line biosensor for the detection of putative toxicity in water contaminants, Talanta 132 (2015) 583–590.
[31] H.L. Tang, et al., In vivo CaspaseTracker biosensor system for detecting anastasis and non-apoptotic caspase activity, Sci. Rep. 5 (2015) 9015.
[32] M. Torkzadeh-Mahani, et al., Design and development of a whole-cell luminescent biosensor for detection of early-stage of apoptosis, Biosens. Bioelectron. 38 (1) (2012) 362–368.
[33] Y. Wang, et al., Bacterial whole-cell biosensors for the detection of contaminants in water and soils, Methods Mol. Biol. 1096 (2014) 155–168.
[34] H. Mano, et al., Development of novel bioluminescent sensor to detect and discriminate between vitamin D receptor agonists and antagonists in living cells, Bioconjug. Chem. 26 (10) (2015) 2038–2045.
[35] S.B. Kim, Fabrication of bioluminescent capsules and live-cell imaging, Methods Mol. Biol. 1098 (2014) 117–125.
[36] S.B. Kim, Y. Ito, M. Torimura, Bioluminescent capsules for live-cell imaging, Bioconjug. Chem. 23 (11) (2012) 2221–2228.
[37] G.S. Baird, D.A. Zacharias, R.Y. Tsien, Circular permutation and receptor insertion within green fluorescent proteins, Proc. Natl. Acad. Sci. U. S. A. 96 (20) (1999) 11241–11246.
[38] T. Nagai, et al., Circularly permuted green fluorescent proteins engineered to sense Ca2+, Proc. Natl. Acad. Sci. U. S. A. 98 (6) (2001) 3197–3202.
[39] R. Sakaguchi, et al., A single circularly permuted GFP sensor for inositol-1,3,4,5-tetrakisphosphate based on a split PH domain, Bioorg. Med. Chem. 17 (20) (2009) 7381–7386.
[40] B. Shui, et al., Circular permutation of red fluorescent proteins, PloS One 6 (5) (2011) e20505.
[41] Q. Yin, et al., Caspase-9 holoenzyme is a specific and optimal procaspase-3 processing machine, Mol. Cell 22 (2) (2006) 259–268.
[42] M.L. Wurstle, M.A. Laussmann, M. Rehm, The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and activity on the apoptosome, Exp. Cell Res. 318 (11) (2012) 1213–1220.