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Fluorescent Coelenteramide-Containing Protein as a Color Bioindicator for Low-Dose Radiation Effects

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Abstract

The study addresses to application of fluorescent coelenteramide-containing proteins as color bioindicators for radiotoxicity evaluation. Biological effects of chronic low-dose radiation are under investigation. Tritiated water (200 MBq/L) was used as a model source of low-intensive ionizing radiation of beta type. 'Discharged obelin', product of bioluminescent reaction of marine coelenterate *Obelia longissimi*, was used as a representative of the coelenteramide-containing proteins. Coelenteramide, fluorophore of discharged obelin, is a photochemically active molecule; it produces fluorescence forms of different color. Contributions of 'violet' and 'blue-green' forms to the visible fluorescence serve as tested parameters. The contributions depend on the coelenteramide' microenvironment in the protein, and, hence, evaluate distractive ability and toxicity of radiation. The protein samples were exposed to beta radiation for 18 days, maximal dose accumulated by the samples was 0.28 Gy being close to a tentative limit of a low-dose interval. Increase of relative contribution of 'violet' fluorescence under exposure to the beta irradiation was revealed. High sensitivity of the protein-based test system to low-dose ionizing radiation (to 0.03 Gy) was demonstrated. The study develops physicochemical understanding of radiotoxic effects.

Key words: fluorescent protein, coelenteramide, discharged photoprotein obelin, multicolor bioindicator, radiotoxicity

Abbreviations:

CLM – coelenteramide, N-[2-benzyl-6-(4-oxocyclohexa-2.5-dien-1-ylidene)-1H-pyrazin-3-yl]-2-(4-hydroxyphenyl) acetamide

CLM-CFP – coelenteramide-containing fluorescent protein

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1. Introduction

Coelenteramide-containing fluorescent proteins (CLM-CFP) are convenient systems for study physicochemical mechanisms of toxic effects. Structural components of fluorescent proteins are polypeptide and aromatic fluorophore. Fluorophore of CLM-CFPs is coelenteramide (CLM) molecule, which is bonded non-covalently with polypeptide inside its hydrophobic cavity. The CLM-CFPs are known to be products of bioluminescent reactions of marine coelenterates (jellyfishes, polyps, etc.). In the course of these reactions, 'photoproteins' (complexes of polypeptides with 2-hydroperoxycoelenterazine) are reconstructed ('discharged') by addition of Ca^{2+} resulting in light emitting. This is why the CLM-CFPs are called 'discharged photoproteins', and this term is commonly used in scientific literature. Unlike green fluorescent proteins, the CLM-CFPs are not so widespread in biomedical investigations and their potential as color biomarkers is currently underestimated.

Fluorescence spectra of CLM-CFPs are wide and asymmetric; they include several components corresponding to different forms of CLM [1-3]. Chemical structures of neutral and ionized forms of CLM are presented in Fig.1. Contributions of these forms to the overall fluorescence spectrum can change. These changes are concerned with photochemical activity of CLM, namely, proton transfer from phenolic CLM group to proton-acceptor, aminoacid residue His22 (Fig.1).

<Fig.1>

Destructive exposures can change protein structure, interatomic distances in CLM surrounding, and hence the efficiency of proton transfer in the CLM electron-excited states. These processes are followed by the redistribution of neutral ('violet') and ionized ('blue-green') forms of CLM. This feature makes CLM-CFPs as perspective multicolor biomarkers for external exposure evaluation. As a result, the CLM-CFPs present a basis for the new type of toxicity assay, i.e. fluorescent bioassay with color registration. Additionally, application of CLM-CFPs forms a physicochemical approach to understanding biological responses to toxic impacts.

It is known that preparations of coelenterate' proteins (photoproteins) are already applied as bioluminescent markers to monitor intracellular calcium [4,5]. CLM-CFPs are products of these bioluminescent reactions, and their application prospects as toxicity bioassays attach multi-functionality to the coelenterate' protein preparations.

Changes of fluorescence CLM-CFP spectra under exposure to organic compounds have been already studied previously [6.7]; a series of alcohols and DMSO were taken here as examples of the exogenous compounds. Temperature-dependent variations of CLM-CFP fluorescence spectra were presented in [8].

Variability of CLM-CFP spectra under radioactive exposure has not been studied yet. Current paper aimed at correlations between fluorescence characteristics of CLM-CFPs and parameters of low-dose radioactive exposure. Discharged obelin, product of bioluminescent reaction of marine coelenterate *Obelia longissima*, has been chosen here as a representative of CLM-CFPs. Dependence of contributions of colored components in the discharged-obelin fluorescence vs. time of exposure to low-intensive ionizing radiation of beta type was under investigation.

2. Materials and methods

The recombinant preparation of photoprotein obelin from hydroid polyp *Obelia longissima* was used to construct a discharged-obelin-based assay system. It was obtained from Photobiology laboratory, Institute of Biophysics, SB RAS, Krasnoyarsk, Russia [9]. EDTA was from Sigma, Germany, Tris and ethanol – from Fluka, Switzerland.

Radioisotope tritium was used as a source of ionizing radiation. Tritiated water (radiochemical purity 98%), was added to the obelin solutions. Characteristics of the samples tested: 200 MBq/L specific radioactivity, and 10^{-5}M obelin concentration. Overall time of exposure to tritium was 18 days; t = 5°C.

The fluorescent spectra were registered in 24 h, at 20°C, with PerkinElmer LS55 fluorescence spectrometer (USA). The parameters of registration were the following: the 360-650 nm wavelength scanning range with 350 nm photoexcitation.

Fluorescence yields Q were calculated in the coordinates: fluorescence intensity-wavelength number. They were compared to those for control (non-irradiated) samples at the corresponding time of radioactive exposure; relative quantum yields Q^{rel} were calculated and plotted vs. time of exposure to tritium. Experimental error was 8-10% for all Q^{rel} values.

Mathematical processing of the complex fluorescence spectra was performed using software packages *Origin 8.5.1* and *Matlab 8.0*. To determine the number and maxima of the spectral components, the second derivative method was used. The spectra were deconvolved into individual Gaussian components in the coordinates: fluorescence intensity – wavelength number [10]. The deviation d of the calculated spectrum from the experimental one was evaluated as follows:

$$d = \frac{\left|S_{\exp} - \sum S_{comp}\right|}{S_{\exp}} \cdot 100\,\%, \qquad (1)$$

where S_{exp} is the area of the experimental spectrum, and S_{comp} is the area of the individual spectral component. The value of d did not exceed 0.5%.

Contribution W of the 'violet' or 'blue-green' spectral components (I or II+III, respectively), to the overall fluorescence spectrum was calculated as follows:

$$W = \frac{S_{\text{comp}}}{\sum S_{\text{comp}}}, \qquad (2)$$

The average values of W were obtained in four parallel experiments with five measurements for all irradiated and control (non-exposed) discharged-obelin solutions. Dependencies of W on time were determined in radioactive and control solutions. Time-courses of the spectral contributions in radioactive solutions were corrected according to those in the control samples. Relative spectral contributions W^{rel} were calculated and plotted vs. time of exposure. Experimental error for W^{rel} values was 8-10%.

3. Results and Discussion

Effects of beta radiation of tritium, a component of tritiated water, on light-induced fluorescence of discharged obelin were studied. The protein samples were exposed to beta-radiation for 18 days, maximal dose accumulated by the samples was 0.28 Gy. This dose value is close to a tentative limit of a low-dose interval.

The 18 day experiment resulted in 80% decay of the fluorescence intensity in the control samples at 5°C. As compared to 40°C [8], lower temperature increases time stability of discharged obelin.

Noticeable changes of the spectral shape were found in all irradiated samples, as compared to the non-radiated (control) samples. Fig 2 presents an example of this change.

<Fig 2>

It was found that all the spectra were a superposition of three components with spectral maxima at 415, 500, and 565 nm, corresponding to violet, blue-green, and green spectral regions, respectively. An example of discharged-obelin spectrum and its components I-III are presented in Fig. 3.

<Fig. 3>

The components were attributed to different fluorescent forms of protein-bound coelenteramide: neutral (I) and ionized (II and III) forms according to data from [11-13]. Ionized forms II and III might differ in effective proton location between phenolic CLM group and His22 as a proton acceptor [14], Fig.1.

Time-courses of the spectral component contributions were studied. Values of relative contributions, W^{rel}, are presented in Fig. 4.

< Fig. 4>

The Figure demonstrates that the exposure to tritiated water results in the increase of contribution (W^{rel}>1) of violet fluorescence (component I) and the decrease of contribution (W^{rel}<1) of blue-green fluorescence (components II and III). The increase of the 'violet' contribution (I, Fig.4) was more than 50% after 18-day exposure. Valuable changes of W^{rel} were observed already after 2-day exposure; corresponding radiation dose absorbed by this time was c. 0.03 Gy. Hence, the fluorescence of discharged obelin demonstrated high sensitivity to low-dose radiation of tritium.

As discussed in [6-8], rise of 'violet' contribution in the fluorescence spectra of CLM-CFPs is an evidence of destructive exposures – chemical or thermal. Radioactive exposure can be considered from the same point of view: it results in partial protein destruction, change of CLM microenvironment, and efficiency of its ionization.

Dependence of the overall fluorescence of discharged obelin on the time of exposure to ionizing radiation of tritium was studied as well. Fig. 5 shows changes of relative fluorescence quantum yields Q^{rel} under exposure to tritium beta radiation during 18-day observation. Moderate time decay of Q^{rel} is evident from this figure. This result shows that overall quantum yield of visible fluorescence can hardly be applied to monitor radiotoxicity of ionizing radiation in water media.

<Fig. 5>

4. Conclusion

The study develops physicochemical aproach of radiotoxic effects. Simplest biological object, coelenteramide-containing fluorescent protein, was applied as a test system. Changes in the protein visible fluorescence spectra were observed under the conditions of low-intensive radioactive exposure: increase of 'violet' and decrease of 'blue-green' fluorescent contributions were found. The effect was explained with destructive ability of low-dose radiation, change of the coelenteramide microenvironment and, hence, decrease of efficiency of photochemical proton transfer in favor of the neutral ('violet') coelenteramide form, Fig.1.

The study addresses the biological effects of chronic low-dose radiation. Prospects for application of the coelenteramide-containing fluorescent protein as a radiotoxicity multicolor bioassay were shown. High sensitivity of the protein response to low-dose ionizing radiation of tritium was demonstrated. Simple registration of luminescent test parameter imparts convenience and prospectivity to the protein-based test system.

Development of the protein-based colored biomarkes can form a physicochemical basis for understanding biological response to toxic exposures in luminescence assay systems of different complexity [15-22].

Acknowledgement

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No conflict of interest

Informed consent

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REPLY TO REVIEWERS:

The text was changed according to the recommendations of reviewers. Type of manuscript was changed to Rapid Communication.

Referee A:

… However, "bioassay", which is typically conducted to measure the effects of an exposure or toxicant on a living system, is far too high for the present basic investigation. It covers one simple experiment at a single condition providing some basic yet novel information. It is recommended to skip the term "bioassay".

Reply: We skipped term "bioassay". In several positions we changed it for "bioindicator" or "test system"

The concept has clearly been described. However, the text is hard to read due to awkward and redundant phrasings as well as unexplained abbreviations.

Reply: Text was corrected

It is highly recommended to shorten the introduction by half, at least, being inproportionate regarding results and conclusions. Preferably, the first two chapters of the introduction may be deleted.

Reply: First two chapters were removed. As well as several sentences along the text. Conclusion was reconstructed.

35: "of a new type bioassays" – please, revise.

Reply: Removed

71: Exactly the same figure as Fig. 1 has already been published in 2016; reuse is often not allowed and may not reconcile with good scientific practice. In addition, the figure is hard to understand, due to an insufficient legend and for readers being not familiar with these processes.

Reply: We removed Yablonnsky diagrams from the figure but left chemical structures.

99: The abbreviation for tritium should consequently be used following its introduction. It should be rather "exposure" than "exposition".

Reply: Sign of tritium (^{3}H) was removed as it was not applied further.

106: Decomposition means degradation; I feel that you mean "deconvolution".

Reply: Corrected

114-116: The sentence and the figure should be transferred to "Results".

Reply: Transferred, along with Fig.

119: I feel that "100%" is missing.

Reply: It is not important here, as we further used relative units of W (W^{rel}). Presentation of W values as fractions is possible, too.

120: The time intervals should be added.

Reply: We added "in 24 h" in line 81.

126: It should be "Results and discussion".

Reply: Corrected

127: The abbreviation HTO may be skipped; it is used only two times. It should be mentioned how often measurements were scheduled.

Reply: Corrected and mentioned " in 24 h" in line 81.

133: This sentence has already been mentioned (see 107-110); it should be deleted.

Reply: Removed.

150: This sentence has already been mentioned (see 129).

Reply: Removed.

165-171: These are no conclusions based on the results from the present study.

Reply: We reconstructed Conclusion

176-178: These 2 sentences belong to the study design; they are redundant in nature.

Reply: Removed.

References are sufficient but may be excessive with regard to significance of results and the length of discussion.

Reply: Amount of references was reduced to 22.

Reference 42 has been submitted but has not been published.

Reply: Removed.

Fig.2 and 3: r.u. should also be given in full.

Reply. Corrected

Fig.3 and 4: HTO should be given in full.

Reply: Figure captions were corrected

Fig.4: The error bars appear to be truncated; it seems that error bars of I and II+III are all of the same length, respectively. Is this really the case?

Reply. The error bars were corrected. We indicated that experimental error for W^{rel} was 8-10%in all experiments (l.102)

Fig.5: It seems that error bars of I and II+III are all of the same length, respectively. Is this really the case?

Reply. We introduced the sentence: (l.85-86): "Experimental error was 8-10% for all Q^{rel} values." As is seen, experimental errors for Q^{rel} were close in all experiments.

Referee C:

1. It would be relevant to provide more information about the time course of spectral components in the control (non-irradiated) samples.

Reply: Information was presented: 100-101, 109-110.

2. A comparison with the high-temperature time course of the spectral data that was studied previously (Alieva et al., 2013) would be interesting.

Reply: 109-110: The 18 day experiment resulted in 80% decay of the fluorescence intensity in the control samples at 5°C. As compared to 40°C [8], lower temperature increases time stability of discharged obelin.

3. I would suggest to revise the first sentence of the second paragraph of Introduction (page 2) as follows: "The important group of experimental methods applied in toxicology utilizes either luminescence properties of toxicants [8] or luminescence response of the pertinent bioassay systems [9]."

New references:

8. Fedorova GF, Menshov VA, Trofimov AV, Tsaplev YuB, Vasil'ev RF, Yablonskaya OI. Chemiluminescence of cigarette smoke: Salient features of the phenomenon. Photochem. Photobiol. 2017. doi: 10.1111/php.12689

9. Roda A, Guardigli M, Analytical chemiluminescence and bioluminescence: Latest achievements and new horizons. Anal. Bioanal. Chem. 2012; 402:69-76. doi: 10.1007/s00216-011-5455-8

Reply: We removed the second paragraph, the references were added (Refs 15-16}

Figure Captions

Fig. 1 Chemical structure of coelenteramide molecule. Neutral and ionized forms.

Fig. 2 Fluorescence spectra of discharged obelin. 1 – control sample, 2 – in tritiated water, 200 MBq/L, 18-th day of exposure.

Fig. 3 Components (I, II and III) of discharged obelin fluorescence spectrum.

Fig. 4 Relative contributions, W^{rel}, of components I and II+III to the fluorescence spectra of discharged obelin in tritiated water, 200 MBq/L. Spectral components I, II and III are shown in Fig. 3.

Fig. 5 Relative quantum yields of discharged obelin fluorescence, Q^{rel}, vs. time of exposure to tritium. Tritiated water, 200 MBq/L.

Fig.1

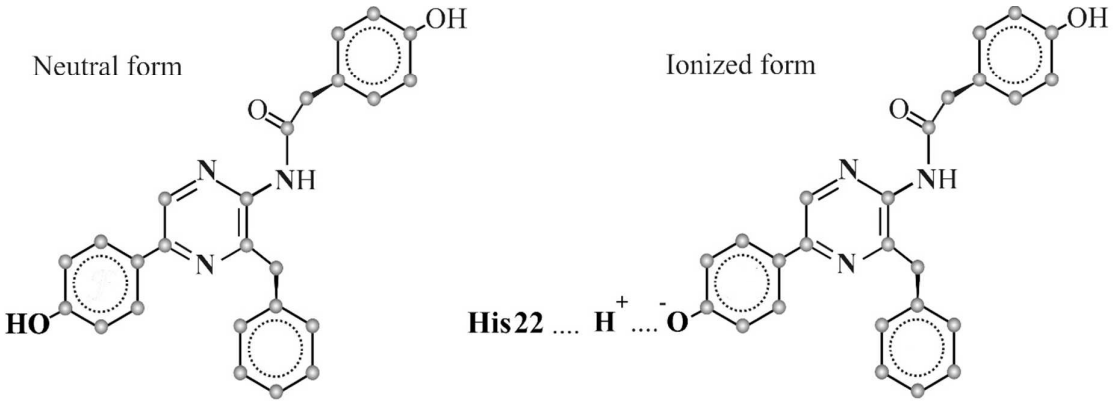


Fig.2

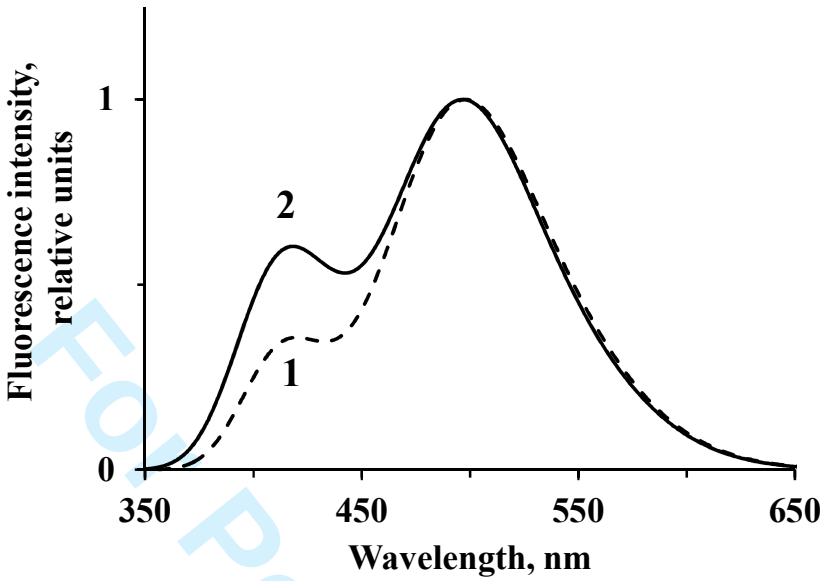


Fig. 3

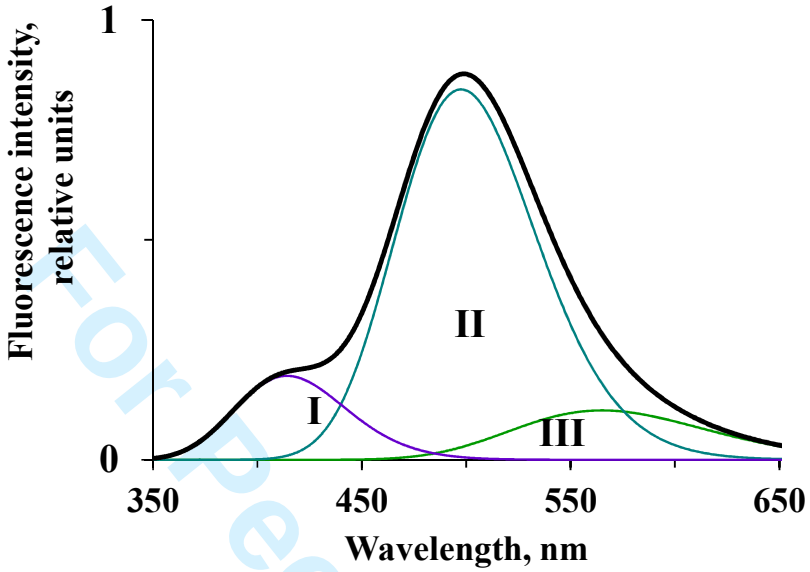


Fig. 4

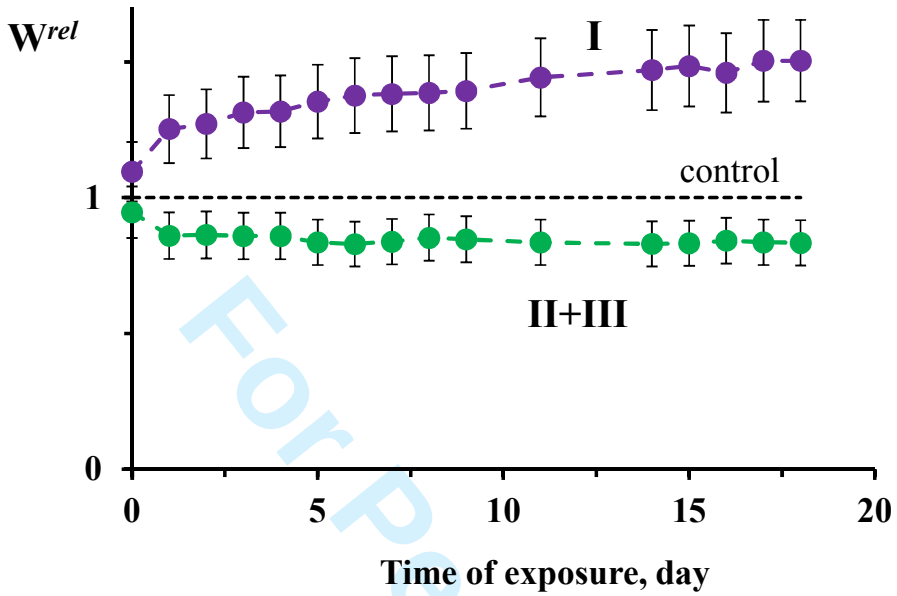
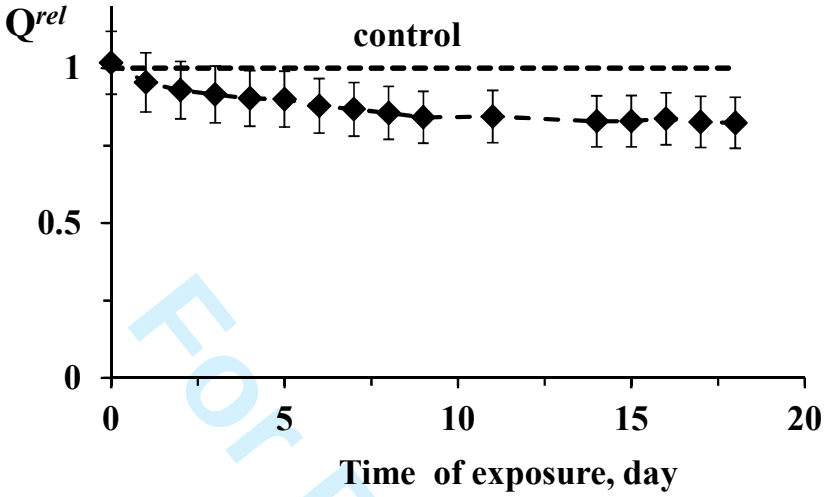
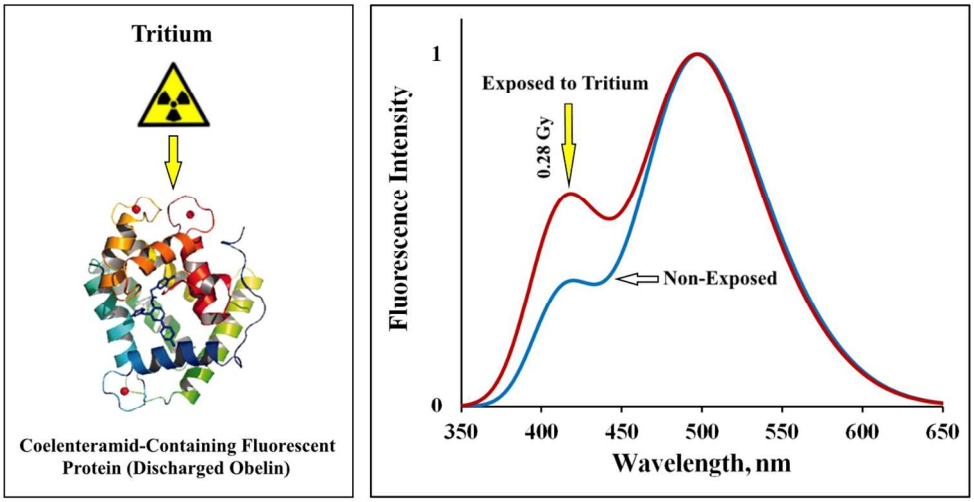


Fig. 5





graphical abstract

372x195mm (96 x 96 DPI)