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Biomarker Discovery and Biotherapeutics Applications of Photosynthetic Light-Harvesting and Bioluminescence Light-Emitting Chromophore-Protein Complexes in Stem Cell Biology and Regenerative Medicine

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Abstract

We have since the 1970's embarked on the development of biologically derived fluorophore-protein complexes that will find applications in the communicable and non-communicable disease etiology processes and their cures. We have since then become largely successful in these endeavors along with interspersed contributions also from investigators who have generally restricted to working in confined disciplines. Their encompassment with our works as this investigator has traversed his definitely chosen and not merely a circumstantial, coincidental, or accidental step-wise multi-disciplinary scientific path from biophysics to regenerative medicine spanning these lines of investigations for last four decades have finally yielded the much necessitated disease related applied biological interventions for human benefits. Taking a cue from our early investigations and findings on which we call attention to the identification and characterization of the use of the primary light-emitting lumazine precursor of riboflavin which is 6,7-dimethyl-8-ribityl lumazine-protein complex which we had derived from the bioluminescence bacterium (*Photobacterium phosphoreum*) wherein it functions as a naturally occurring fluorescence light emitter (LumP). These *in vivo* phenomena have been a precursor to the subsequent developments *in vitro*. This *in vivo* to *in vitro* investigation path of ours has been also comprised among others of binding of photosynthetic light-harvesting marine dinoflagellate algae (*Glenodinium sp*.) derived peridinin-chlorophyll *a*-protein (PerCP) complex-labeled monoclonal antibodies useful in the development of flow cytometry. These fluorescence labeled antibodies bound antigens which include those of communicable infectious diseases (HIV/AIDS – *env*-gp160, *gag*-p24), non-communicable but also potential hereditary and malignant disorders (Cancer/Tumor Markers – Melan-A/Mart-1 of melanoma), normal immune response cells (Human/Mouse/species cellular MHC/TCR/CD45/CD33/CD56/CD19/CD41), and of types of stem cells (CD34/CD38/c-Mpl/Oct4/Neuropilin-1/SOX17). Such antigens have been analyzed by us and other investigators by fluorescence-activated cell sorting (FACS – cell surface and intracellular binding), confocal

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fluorescence microscopy, or/and immunohistochemistry, to determine qualitative and quantitative antigen expression levels and their mechanistic implications. We have followed stem cell differentiation patterns and signaling mechanisms through marker antigen-antibody binding wherein the antibodies are labeled with covalently linked fluorophore-protein complexes or fluorescence emitting chromophores. These complexes among others also have included PerCP. We are also now in the process of developing flow cytometry applications of additional visible light emitting chromophore-protein complexes through industrial collaborations.

Keywords: Peridinin, Chlorophyll, Lumazine, Riboflavin, Excitons, Fluorescence

Introduction

Our and other investigators' work in the field of photochemistry and photobiology in the 1970's [1-4] had begun to lay the ground breaking for eventual easy detection of antigens through multi-color fluorescence labeling of polyclonal and monoclonal antibodies that bind to various target antigens on the mammalian cell surface [5]. The light-harvesting pigments of different species go through a series of intracellular pathways which convert the energy from the photons absorbed into intra-cellular molecular reactions and extra-cellular interactions such as for example in the photosynthetic lamellae of the photosystems I and II. The chromophore topography of the photosynthetic light-harvesting peridinin-chlorophyll *a*-protein complex [PCP subsequently designated or abbreviated by others as PerCP (Per = peridinin, C = chlorophyll *a*, P = protein/peptide)], as we had first reported [1], absorbs visible light energy at the wavelength maximum of 483 nm [2]. The PerCP fluorescence emission (676 nm) coincides with that of chlorophyll *a* existing on the same protein all together as a complex of molecules naturally including the amino acid chain macromolecular protein entity. The reported ~picoseconds magnitude energy transfer rate must be sufficient to prevent a loss of energy through "external" cellular microenvironment within the species and for efficient transfer to the chlorophylls to utilize this energy in the photosynthetic photosystems. Similarly, the light-emitting bioluminescent bacterium utilizes visible light (417 nm) absorbing molecule in the cellular metabolic pathways such as the riboflavin precursor, lumazine, and emit blue fluorescence light at 475 nm when in complex with a protein, lumazine-protein [3]. However, what differentiates our findings on PerCP and LumP is that in the former, the cellular signaling mechanisms occurs post-absorption of light via peridinin. In the latter, the relevant but more likely a coincidental cellular signaling mechanism when a lumazine is present occurs pre-absorption of internally generated biological reactions energy into that of light energy within the bacterium species and thus confers the bioluminescence trait. This characteristic presumably helps the bacterium to scare away predators or cause female-male sex/gender attraction between among their hosts such as marine fish in the sea bed. Their involuntary role in nature could be to populate specific sites of a host such as a type of marine fish that may carry "unconventional receptors" which have a binding choice to bioluminescent bacterium on the host's external body as part of the evolution process. Both peridinin and lumazine serve the dual functions. In the case of peridinin it serves as light-harvesting carotenoid molecule and also prevents the chlorophyll from photodynamic damage [6] as in *Glenodinium sp*. The lumazine is responsible for both the necessity of the species *Photobacterium phosphoreum* in being a riboflavin overproducer through serving as a substrate for riboflavin synthetase and also as a light-emitter again in the form of a complex with its non-covalent binding to a protein macro molecule [3]. Such binding of lumazine to a designated "carrier" protein could also have prescribed roles to cause functional interactions with the enzyme riboflavin synthetase.

While PerCP has been adopted for labeling antibodies, Lum and LumP have been set aside to this juncture in fluorescence labeling techniques due to their near blue light absorption proximity to its fluorescence emission wavelength. Further the probability of detachment of Lum from its natural non-covalent linkage or anchor to intra-species expressed complex P (Protein/Peptide) which may also carry the promotion of enzymatic function via interactions with riboflavin synthase. This however does not preclude the 6,7-dimethyl-8-ribityl lumazine molecule alone minus the *in vivo* carrier protein, from being tagged by itself as a fluorescence label to the antigen binding monoclonal antibodies, as is the case

with phycoerythrin (PE) and allophycocyanin (APC) fluorophore molecules. LumP has probably been considered a technically and practically an overlapping challenge in developing or generating the cut-off filters for the relevant lasers in the manufacture of FACS instrumentation. Phycoerythrin (PE) [4] has been preferred in the technically challenging "competition" when compared to lumazine. However, technical challenges have been proven to be overcome with the potential necessity for refinement which in the case of blue light emitters like lumazine, may lead to further technology development of lasers of ever narrowing wavelength widths for micro-laser treatments. Such techniques could be expected to be useful both in pathologically required as well as cosmetically selective surgeries in the future with advancing technologies. Thus in the case of PerCP, complexation of this peridinin-chlorophyll *a*-protein complex to monoclonal antibodies has been achieved for usage in FACS analyses suggesting that any manufacturing technological impediments could be overridden by experimental and clinical necessities and requirements.

External Applications of Chromophores

Biologically occurring chromophores could well be considered a gift of nature for tissue and biomedical engineering such as the fluorescence activated cell sorting (FACS) also known as flow cytometry wherein the fluorophore labeled antibody-antigen complexes are analyzed *in vitro*. These chromophores are also used in confocal fluorescence microscopy and immunohistochemistry.

Evolutionary Chromophore Binding

The photosynthetic accessory pigments of the prokaryotic blue-green algae and the eukaryotic red algae such as *Schizothrix calcicola* and *Porphyridium cruentum* carrying the respective chromophores C-Phycoerythrin (PE) and R-Phycocyanin transfer energy to chlorophyll *a* in a step-wise manner. This light-harvesting process consists in general of fluorescence emission of the lower wavelength energy donors and their excitation for energy transfer *in vivo* to the biologically designated higher wavelength energy acceptors in the vicinity or part of the photosynthetic lamellae [4]. However, similar energy transfer mediated in the PerCP is independent of such an intra-complex fluorescence emission by peridinin to chlorophyll *a* [1, 2]. This dissimilarity between PE and PerCP is discussed again in this manuscript in the next pages which points out the greater biological intra- to inter- photosynthetic lamellae sequestration of PerCP of the photoreception over a wider wavelength range without suffering a loss of the available received light energy through an intermediate wavelength release due to the lack of peridinin fluorescence emission.

Applications to Antigen Binding

Preferentially and also as a requirement for flow cytometry, a non-detachable covalent binding of these naturally and biologically occurring *in vivo* or *ex vivo* visible light emitters to antibodies of extraneous antigens is needed/ Such antigens are of known and also for the discovery of unknown novel and disease specific characteristic markers or phenotypic proteins. These antigens are detectable by the intrinsic fluorescence of the labeled chromophore (fluorophore) to antibodies. The labeled antibodies bind generally in a non-covalent manner to their biological target antigens wherein the antigen-antibody epitope tertiary structural interactions are normally involved.

Normal and Disease Cell Surface Antigens

Cell surface antigens include markers for normal cells such as the various types of stem cells existing at different stages of differentiation. Diseased cells expressing specific antigens characteristic for different types of tumors are also detectable by characterized antibodies labeled with the fluorescent chromophores. As we had reported these methods are also useful for the detection and characterization of putative new antibodies including such as those isotypes of immunoglobulins generated against HLA Class I and II antigens *in vivo* but assayed *ex vivo* [7].

Biomarkers for Drug Discovery

Detection of novel biomarkers through newly generated hybridomas for putative target specific monoclonal or polyclonal antibodies is much facilitated by this discovery of biologically occurring light-harvesting and light-emitting chromophores characteristic for their fluorescence in the visible light wavelength regions. Such new antibodies are linked covalently with these fluorophores which can then selectively bind to putative biomarkers. Multi-color fluorescence labels of multiple antibodies will assist in the deciphering of functional potency of the target/ed biomarkers in their order of biological significance or relevance.

Discussion

Primary vs Secondary Light Emitters

In a manner dissimilar to which the flavins serve as acceptors of light energy or are involved in light emission from a luciferase mediated reaction, in *Photobacterium phosphoreum* the well characterized lumazine precursor of riboflavin itself is very well expected to be the primary light emitter without involving a flavin molecule considering the emission maximum of 475 nm of lumazine [3]. Lumazine - Protein is the primary light emitter in its characteristic bioluminescence of *Photobacterium phosphoreum.*

The so called "classical" luciferase reaction in which involvement of flavin mononucleotide (FMN) is implicated, which in *Photobacterium phosphoreum* may not hold ground, or is inapplicable for this bacterium. The fluorescence emission wavelength regions are similar for both lumazine protein and FMN and hence the biological need for a luciferase reaction in *Photobacterium phosphoreum* does not seem to arise. Since the chromophore of the yellow fluorescence protein (YFP) has not been identified through a detailed characterization (viz. molecular identification of its chromophore) it is difficult to know whether YFP is a secondary light emitter in *Vibrio fischeri* strain Y-1 [8], or is in fact the primary emitter as LumP is in *Photobacterium phosphoreum*. Discussions on the existence of secondary emitters of bacterial bioluminescence do not necessarily confirm that the light emitters via the classical luciferase reaction exist in all bioluminescent bacteria. Hence the blue fluorescence protein (BFP), LumP, of *Photobacterium phosphoreum* and yellow fluorescence protein (YFP) of *Vibrio fischeri* may possess alternative biologically internally generated or released energy acceptors for light emission. This would then occur without a flavin intermediate either as the primary energy donor to a secondary light emitter or mediator of emission by itself as a possibility which is negated more so in *Photobacterium phosphoreum* by LumP (BFP). The lumazine protein makes the involvement of FMN rather remote as the primary light emitter. The putative chromophore of YFP could have the possibility of FMN being involved as the primary light emitter with its fluorescence being shifted to a higher wavelength due to the internal complex with protein. However since the FMN absorption and LumP fluorescence emission wavelength maxima are in close proximity to each other the primary light emitter in the bioluminescence of *Photobacterium phosphoreum* is expected to be none other than the lumazine protein without the involvement of FMN.

Utilization of ATP/ase in DNA Photorepair

Flavins as photoreceptors in DNA photolyase and the involvement of ATP/ase in DNA repair including the alternately but originally termed DNA photoreactivating enzyme (PRE) which is protected by exposure only to yellow light of external environment [9, 10]. These DNA repair enzymes involve the genes *Vcphr* and *UvrA*, also harvest blue light for cellular biological reactions [11, 12].

Bacteriorhodopsin found to be analogous to human rhodopsin absorbs visible light at a maximum around 570 nm [13, 14]. Photophorylation in *Holobacterium halobium* incorporating mitochondrial ATPase could well be similar to the involvement of ATP/ase in DNA repair in *Escherichia coli* [10, 11, 13, and 14].

Difference in Energy Transfer Mechanisms of PerCP and PE

The light-harvesting with respect to PerCP of the marine dinoflagellate algae encompasses the entire blue light wavelength region even in the absence of absorption of green light due to the highly efficient picosecond rate light energy transfer to chlorophyll *a* without energy loss through fluorescence in the intermediate green-yellow region that occurs between blue and red wavelengths [1]. Thus *Glenodinium sp.* and the other dinoflagellate algae expressing PerCP are at variance with that of PE expressing species due to the presence the carotenoid, peridinin, in dinoflagellates.

In contrast, the light-harvesting mediated by phycoerythrin (PE) / phycocyanin absorption maximum of ~545-565 nm do not harvest the blue wavelength region [4]. Thus PerCP is biologically inherited or conferred with a light-harvesting process that is of a greater efficiency over a wider wavelength region due to its characteristic absence of loss as green-yellow fluorescence energy. Also this intermediate and intermittent rapidly "travelling" energy through the designated or delineated visible light receiving photon mediated pathways is sequestered through a direct picosecond rate of energy transfer to the chlorophyll *a* excited states by peridinin (carotenoid) excitons [1, 2]. This sequestration of occurs across the wide spectrum of visible light energy from blue to red wavelengths via the green and yellow regions as well. The existence of such an artificial biological "rainbow" within the light-harvesting *Glenodinium sp.* seems to be the hallmark of this particular species of marine algae.

Applications of Photobiology to Tissue and Biomedical Engineering

Hence the usefulness of PerCP as a widely applicable fluorescence label of monoclonal antibodies for FACS analyses has had its origins of the description of the mechanism of picosecond rate energy transfer within the PerCP "macro"-molecular biological entity dating around 40 years back to our work in the 1970's [1, 2]. The far reaching developments and the subsequent results and applications of the initiation of this work have enabled their utilization over the years to our ensuing studies on infectious diseases [15-19] and etiologies of cancer [20, 21] and in the use of the chimeric humanized mouse model systems *in vivo* as well as *ex vivo* [22-27]. This long process has seen applications to the design and manufacture of instrumentation such as for flow cytometry for the labeled fluorophore (fluorochrome) analyses of cellular antigens and the applications for detection of multiple antigens on single cells using simultaneous multi-color fluorescence analyses and including the confocal spectroscopy, and immunohistochemistry. The *vice versa* between development of the instrumentation for translational medicine (biomedical engineering), and tissue engineering which have led to the devices for basic experimental research, are inextricably linked.

Relevance to Regenerative Medicine

Regenerative medicine comprises of the use of different types of stem cells which express antigens characteristic of the cell type that exists at a particular differentiation stage [28]. These antigens or markers are qualitatively or quantitatively analyzed by fluorescence techniques and methods including flow cytometry using cell type specific antigens. The cells are sorted and/or purity estimated through FACS analyses and then used for transplantation into the diseased organ sites of damage or degeneration for the therapeutic treatment process of regeneration [29, 30]. The autologous or allogeneic stem cells utilized for regeneration may as necessary have undergone external genetic or other manipulations prior to implantation. Examples of such as these include genetic engineering *in vitro*, stromal reconstitution *in vivo*, and apheresis to obtain re-transplantable CD34+ hematopoietic progenitor stem cells *ex vivo* as in autologous bone marrow transplantation. Degenerative conditions of various organs do occur and it is to be expected that future basic and clinical translational research and development will necessarily involve the discovery of novel biomarkers and the use of bioinformatics, aided by experimental use of FACS instrumentation. This will require the development of newer labels of fluorescence emitting

molecules such as the yet to be employed lumazine fluorophore.

Conclusion

These long and enduring scientific journey endeavors of mine/ours beginning in the 1970's has witnessed the tracing of fascinating intra-, inter-, and extra-biological processes. Undoubtedly, it has been an unforgettable experience having been associated with science carrying inevitable applications to current translational medicine. Even though specialized disciplines of sciences and medicine have arisen as a human made "practical evolution", physiologically it is inherent in the nature's biological evolution itself that has caused the erasing of the delineated scientific subject boundaries. The spanning of such subjects ranging from biophysics to bioinformatics has made the crossing and overlapping of the concepts involved in these boundaries that have led to multi-disciplinary investigations.

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