Fluorescent Probes

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Bilirubin in a New Light**

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Bilirubin (BR), a metabolite of heme, is usually associated with human waste, the color of which it largely determines. It is also responsible for the visible symptoms of jaundice, which occurs when the clearance of bilirubin from the body is slow. This is indeed a considerable chemical challenge, since bilirubin is quite lipophilic by virtue of a network of intramolecular hydrogen bonds that tie up its polar functionalities (Scheme 1). Only after enzymatic conjugation with glucuronic acid is this network broken, and conjugated bilirubin (C-BR) can be readily excreted. Unconjugated BR, on the other hand, tends to accumulate in adipose and nervous tissues, which can have toxic effects, particularly in in newborns. Thus, a reliable quantification of BR in serum is crucial for assessing the severity of neonatal jaundice.

To date, the quantification of bilirubin relies on a colorimetric method devised by the physician Albert Abraham Hijmans van den Bergh nearly one-hundred years ago.[1] It was found that C-BR readily reacts with an aqueous solution of diazotized sulfanilic acid to yield products that can be quantified by absorption spectroscopy. By contrast, BR requires the addition of solubilizing ethanol to become reactive. Thus, the combined level of both bilirubins, and by subtraction of C-BR the level of toxic BR, can be determined.

Simple as this method is, it is relatively unreliable due to the complexity and variability of the chemical reaction involved and the inhomogeneity of biological samples. A fluorescent assay based on a clean chemical event would be clearly advantageous. It is therefore welcome news that Kumagai et al. recently identified a protein that becomes fluorescent in the presence of BR.[2] This interaction was found to be specific and sensitive enough for the reliable measurement of BR concentrations in human serum and tissue samples.

It all started with a surprising finding in 2009:[3] muscle fibers of the Japanese freshwater eel (*Anguilla japonica*) proved to be fluorescent and a protein was shown to be involved. This protein, termed UnaG, belongs to the fatty-acid-binding proteins (FABP) and has 56% homology with human brain FABP. After cloning and expression in bacteria and HeLa cells, the fluorescence was only detectable in the human cells, suggesting that a cofactor absent in bacteria was needed to turn it on. Furthermore, HeLa cells transfected with UnaG exhibited bright fluorescence under hypoxic conditions, implying that oxygen was not required for the maturation of the fluorophore. Addition of fetal bovine serum (FBS) to purified apo-UnaG turned on fluorescence immediately.

Based on this knowledge the hunt was on for the small molecule responsible. Fluorescence could only be induced when apo-UnaG, heterologously expressed in *E. coli*, was added to fractions of FBS that contained albumin or high-density lipoprotein. Both are known to carry BR. Expression of FLAG-tagged UnaG in HEK cells, purification, extraction with chloroform/methanol, and MS analysis then gave a signal at 585.30 Da, which confirmed BR as the cofactor.

Unconjugated bilirubin itself has an absorption maximum of $\lambda_{Abs} = 450$ nm, accounting for its yellow-orange color, and is only very weakly fluorescent. After addition of apo-UnaG, this absorption maximum shifts to $\lambda_{Abs} = 498$ nm, and strong fluorescent emission is observed at $\lambda_{Em} = 527$ nm (Scheme 2). This property explains the name of this new fluorescent protein, easily remembered not only by Sushi aficionados: UnaG (presumably pronounced "Una-Gee"), a word play on, *una*gi, the eel, and *g*reen, the color of its fluorescence.

Titration studies of apo-UnaG with unconjugated BR provided an extremely low K_d of 98 pM. As such, UnaG outcompetes human serum albumin (HSA), whose K_d with unconjugated BR was determined to be 87 nM, a factor of thousand higher. In addition, HSA is nonfluorescent when bound to BR. Furthermore, addition of other members of the heme catabolic pathway, such as biliverdin (BV), urobilin, C-BR, and ditauro BR to UnaG gave virtually no fluorescence. This extremely high affinity and selectivity of UnaG for BR should enable the measurement of unconjugated bilirubin in biological samples. Indeed, BR levels in human serum samples could be readily determined using UnaG. The reaction was found to be complete after 10–20 min, and the fluorescence level was stable for up to one hour.

UnaG can be genetically fused at its N- and C-terminus with other proteins (such as mCherry in the present study) and easily expressed in a variety of cells. As such, it can serve as a genetically encodable protein tag, like green-fluorescent protein (GFP) and its derivatives,[4] with the important difference that fluorescence can be induced by the ubiquitous or easily added BR. With a fluorescence quantum yield of $\Phi = 0.51$, a molar extinction coefficient of $\varepsilon_{max} = 77300$, and

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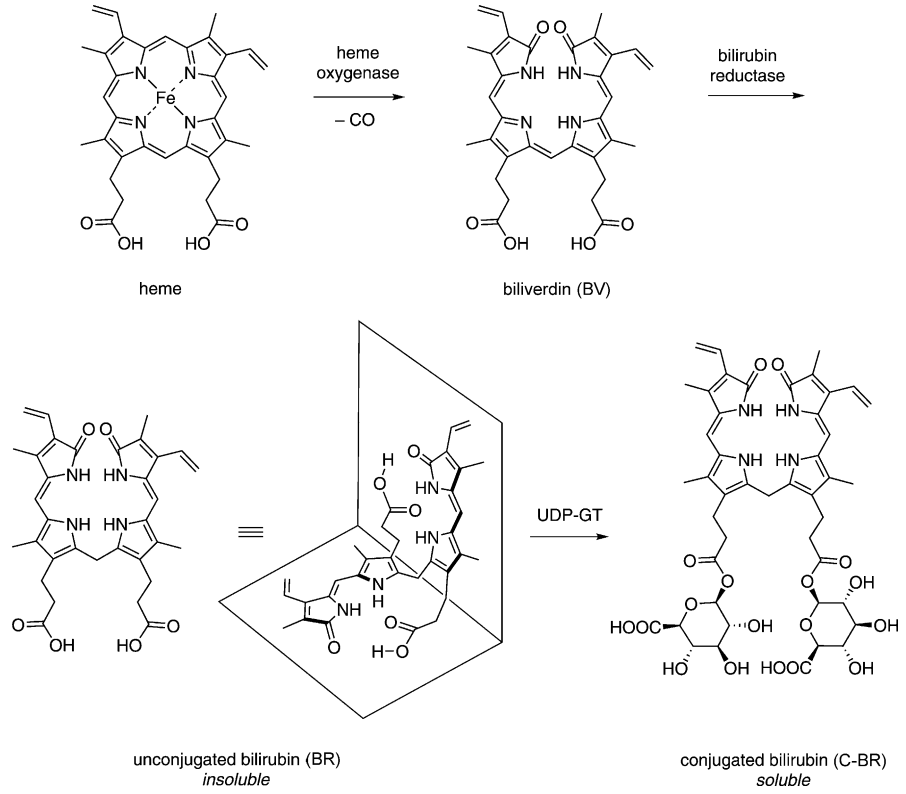
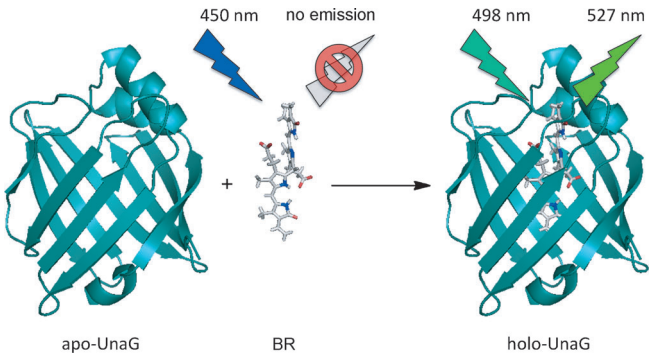


Scheme 1. Heme is enzymatically cleaved to biliverdin (BV), which is then reduced to unconjugated bilirubin (BR). UDP-glucuronosyltransferase (UDP-GT) converts insoluble BR to the water-soluble conjugated bilirubin (C-BR). BR requires the addition of ethanol to become soluble and reactive towards diazotized sulfanilic acid while C-BR readily reacts with an aqueous solution of the reagent.

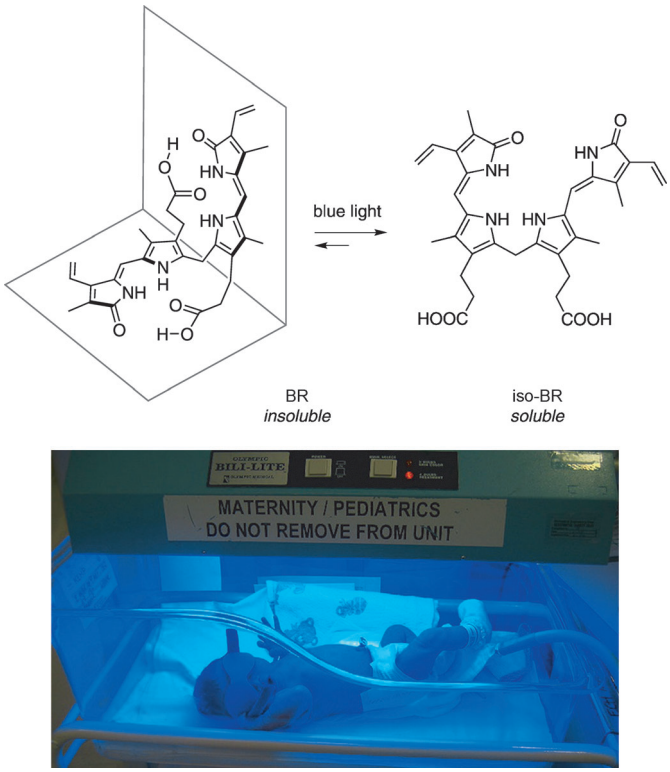


Scheme 2. How UnaG works. The apo form of the protein and unconjugated bilirubin (BR, $\lambda_{Abs} = 450$ nm) are not fluorescent. In the holo protein, the absorption maximum of the chromophore shifts to $\lambda_{Abs} = 498$ nm and emission is observed at $\lambda_{Em} = 527$ nm.

a molecular weight of $M_w = 16.5$ kDa UnaG compares favorably to widely used fluorescent proteins, such as enhanced GFP ($\Phi = 0.60$, $\varepsilon_{max} = 49550$, $M_w = 26.9$ kDa). Although the quantum yield of UnaG is lower, the extinction coefficient is higher and the protein is substantially smaller, which might benefit genetic fusion without disruption of biological function. In addition, UnaG does not require oxygen to become flurescent, which limits the use of GFP and its derivatives under certain, for example, hypoxic, conditions.

Mutational studies showed that the amino acid asparagine 57 (N57) is crucial for the fluorescent behavior of UnaG. When this residue was changed to a glutamine (N57Q) or an alanine (N57A), fluorescence was diminished and completely abolished, respectively. The exact binding mode of BR to apo-UnaG was determined by X-ray crystallography at 1.2 Å resolution (PDB: 4I3B). It was found that N57 is in contact with one of the carboxylates and lactam rings of BR, which is bound in an extended conformation. In the fluorescent protein, the intramolecular hydrogen bonds of free BR have been replaced with hydrogen bonds to water molecules or side-chain residues. X-ray crystal structures of the two mutants mentioned above were also obtained (PDB: 4I3C (N57A), 4I3D (N57Q)). It was not immediately obvious from these structures, however, why the N57A mutant is non-fluorescent and what other mutations should be attempted to shift the excitiation and emission wavelength of UnaG. As in the "fluorescent protein paintbox" derived from GFP,[5] fluorescent color-tuning may require random screening or a directed evolution apporach.

Given the high affinity of UnaG to BR, it is tempting to speculate whether it could be used in therapeutic approaches to treat jaundice, in particular the neonatal variety. Apart from practical limitations and immunological concerns, however, this is not really an issue since a very effective therapy is already available (Scheme 3): Newborns with neonatal jaundice are nowadays exposed to blue light (420–470 nm), which isomerizes a double bond in the BR molecule and breaks the network of hydrogen bonds.[6] This renders the isomer soluble for further metabolic processing and excretion (Scheme 3).



Scheme 3. BR can be converted into a soluble form by simple irradiation with blue light. This phototherapy is widely used in the treatment of neonatal jaundice, either with a blue-lit incubator (shown) or with blankets containing blue LEDs.

Arguably, this is the most useful (and endearing) application of photoswitching in medicine to date.

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[1] A. A. H. van den Bergh, P. Muller, *Biochem. Z.* **1916**, *77*, 90.
[2] A. Kumagai, R. Ando, H. Miyatake, P. Greimel, T. Kobayashi, Y. Hirabayashi, T. Shimogori, A. Miyawaki, *Cell* **2013**, *153*, 1602.
[3] S. Hayashi, Y. Toda, *Fish. Sci.* **2009**, *75*, 1461.
[4] R. Y. Tsien, *Annu. Rev. Biochem.* **1998**, *67*, 509.
[5] R. Y. Tsien, *Angew. Chem.* **2009**, *121*, 5721; *Angew. Chem. Int. Ed.* **2009**, *48*, 5612.
[6] A. F. McDonagh, D. A. Lightner, *Pediatrics* **1985**, *75*, 443.