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Development of new methods for determination of bilirubin

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ABSTRACT

The ever-increasing demand for a sensitive, rapid and reliable method for determination of serum bilirubin level has been inciting the interest of the researchers to develop new methods for both laboratory set up and point of care applications. These efforts embrace measurement of different forms of bilirubin, such as, unconjugated (free and albumin bound) bilirubin, conjugated (direct) bilirubin, and total (both conjugated and unconjugated) bilirubin in the serum that may provide critical information useful for diagnosis of many diseases and metabolic disorders. Herein, an effort has been made to provide a broad overview on the subject starting from the conventional spectroscopy based analytical methods widely practiced in the laboratory setup along with the sophisticated instrument based sensitive methods suitable for determination of different forms of bilirubin to various portable low cost systems applicable in point of care (POC) settings. In all these discussions emphasis is given on the novel methods and techniques bearing potential to measure the bilirubin level in biological samples reliably with less technical complexity and cost. We expect that this review will serve as a ready reference for the researchers and clinical professionals working on the subject and allied fields.

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1. Introduction

Bilirubin ($C_{33}H_{36}N_4O_6$) is a yellow tetrapyrrole compound catabolically produced from heme, which is formed mainly by the degradation of red blood cells (RBCs) in the body. Following its formation in the tissue sites, heme is transported into the liver in an unconjugated form using serum albumin as carrier protein. The dissociation constant of the interaction between this low water soluble compound and albumin is in the range of 108–107 M^{-1} [1,2]. Bilirubin is passively transported into hepatocyte with the help of an organic anion transporter protein-1B1. In the liver, it is conjugated with glucuronic acid by an enzymatic esterification reaction. This esterification with sugar transforms the molecule into a more water soluble form. The conjugation of the sugar moiety to bilirubin is catalyzed by uridinediphosphate glucuronyltransferase-1A1 (UGT1A1) [3]. The water soluble bilirubin is then channelized through kidneys and gut for excretion in urine and faeces, respectively. Bilirubin can be further metabolized into urobilinogen by intestinal microflora. The biochemical pathways of heme to urobilinogen conversion through intermediate bilirubin is briefly depicted in Scheme 1 [4].

The normal concentration of bilirubin in the blood of a healthy person lies in the range of 0.3–1.9 mg dL^{-1} that covers conjugated bilirubin (0.1– 0.4 mg dL^{-1}), unconjugated bilirubin (0.2–0.7 mg dL^{-1}) and a minor amount of non–protein-bound unconjugated bilirubin (free bilirubin) [5]. The chemical structures of different forms of bilirubin are shown in Scheme 2. A low level of serum bilirubin concentration is associated with iron deficiency and coronary artery disease among others; whereas, its higher concentration in serum (>2.5 mg dL^{-1}) would lead to hyperbilirubinemia, which is clinically termed as jaundice. Hyperbilirubinemia is the result of an imbalance between the formation of bilirubin in serum and its excretion from the liver primarily due to hepatocellular disorders, hemolytic diseases, and cholestasis. Usually, the neonates are susceptible to hyperbilirubinemia. In fact, about 50% of term and 80% of preterm babies develop hyperbilirubinemia at an early stage of their birth [6]. The reason may be attributed mainly to the shorter lifespan of RBCs in neonates than the adult which leads to a greater bilirubin burden on the hepatic metabolism. High bilirubin level in the body is efficiently eliminated following the enzymatic conjugation process as discussed above. However, when there is a mutation in the exons coding for UGT the function of the enzyme is impaired resulting in accumulation of a very high level of unconjugated bilirubin in serum. The jaundice developed due to the reason, which is known as Crigler-Najjar syndrome, is apparent at birth or in infancy. The blockage or infection of the biliary tract or congenital disorders of the bile duct (biliary atresia) also cause hyperbilirubinemia. The unconjugated free bilirubin is lipophilic in nature and it can cross the blood-brain barrier. This free bilirubin is therefore, a prime determinant of tissue uptake and toxicity of bilirubin, and plays an important role in the pathogenesis of bilirubin encephalopathy including kernicterus in jaundiced newborn [7]. Hence, in the case of neonatal jaundice, the level of free bilirubin in blood serum is a better parameter to assess the risk caused by hyperbilirubinemia. A large number of babies are at risk globally for neonatal hyperbilirubinemia-related adverse outcomes [8]. The global burden of extreme hyperbilirubinemia and Rhesus disease is disproportionately heavy (11 fold more) for the under developed countries. The serum bilirubin assay is generally performed to test the liver function, which may be impaired due to inflammatory or obstructive lesions. Various methods on bilirubin estimations have been previously reviewed [9,10]. There has been an enormous progress made over the last few years on the development of novel methods and techniques for detection and determination of bilirubin. This review summarizes the overall progress with a focus on the new development on the subject. At the outset, a brief discussion on the major chemical principles that have been conventionally employed for bilirubin estimation is presented.

2. Conventional methods for bilirubin estimation

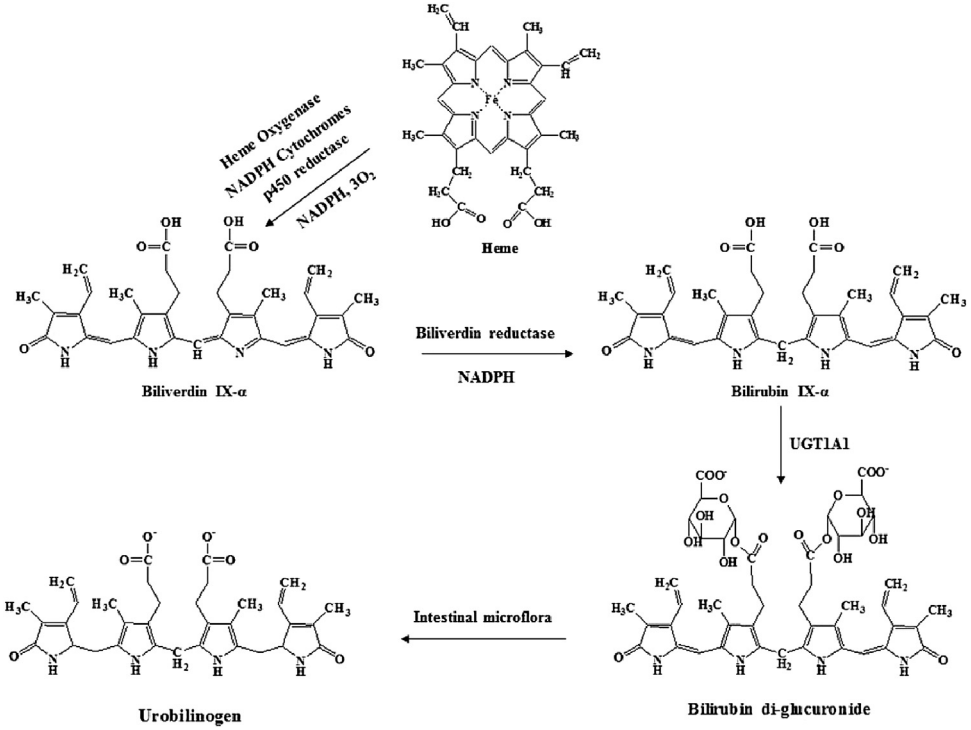
Different conventional methods widely used for the estimation of direct (conjugated), indirect (unconjugated) and total bilirubin in serum have been systematically reviewed in a previous paper [9]. Here, a brief outline on the evolution of the underneath chemical principles involved in two prominent chemical analytical methods, namely, Diazo and Vanadate oxidase methods are presented.

2.1. Diazo method

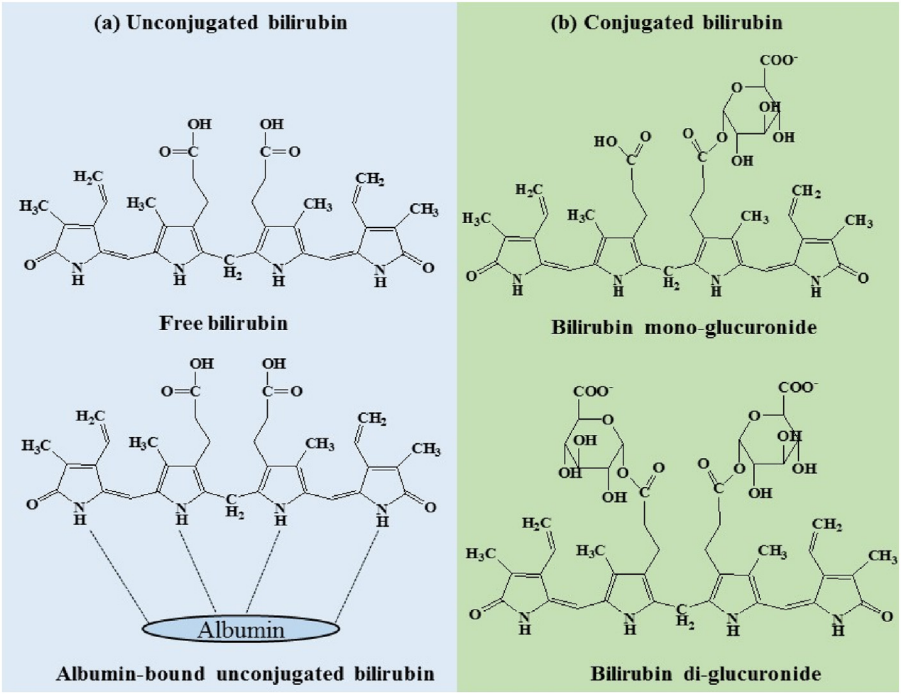
Bilirubin can be quantified as native, derivatized tetrapyrroles, or as its azo-derivatives. The reaction of bilirubin with diazotized sulfanilic acid is known as a diazo reaction (Scheme 3) [10]. The reaction was introduced in 1918 by van den Bergh which led to the widespread adoption of this reaction for quantitative measurement of bilirubin in serum. In this reaction, two isomeric azo pigments with absorbance maxima at $\lambda_{530\,nm}$ are formed. The conjugated bilirubin reacts directly with diazo reagents due to their greater solubility in serum and less affinity for albumin, hence termed it 'direct bilirubin'. The unconjugated bilirubin, on the other hand, needs an accelerator for its solubilization and hence, it is termed 'indirect bilirubin'. A small organic molecule is used as an accelerator to solubilize unconjugated bilirubin and/or displace it from albumin. There have been a number of modifications of the diazo reaction. Even though the normal Ehrlich's diazo method gave result within one minute of reading, not all types of bilirubin could be measured quantitatively. Moreover, the diazo methods are pH sensitive which makes these methods less reliable. Later, Malloy and Evelyn (1937) introduced 50% methanol as an accelerator which reacts with the total bilirubin in the serum to form a pink to reddish-purple colored compound. The absorbance of azodipyrrole was measured at λ_{540nm} to determine the total bilirubin. A mixture of caffeine and benzoate in acetate is used as an accelerator in the diazo reaction for the determination of total bilirubin level. The method involves the addition of alkaline tartrate at the end of the reaction, which shifted the absorbance maxima of the azodipyrrole products from $\lambda_{530\,nm}$ to $\lambda_{600\,nm}$ resulting in the reduction of interference caused by hemoglobin. This method was recommended as a procedure of choice for total bilirubin estimation by the U.S. National Committee for Clinical Laboratory Standards [11]. The advantages of this method are (i) not affected by pH changes, (ii) maintains the optical sensitivity even at low bilirubin concentration, (iii) insensitive to high protein concentrations. Some further modifications of this method involve the substitution of caffeine by dyaphylline, dimethyl sulfoxide, *N*,*N*'-dimethyl formamide, acetamide and sodium dodecyl sulfate. In other methods, ascorbic acid or potassium iodide was added to eliminate excess diazo reagent because excessive diazo reagent in the reaction system results in the decomposition of product.

2.2. Vanadate oxidase method

This method is based on the oxidation process of bilirubin to biliverdin using vanadic acid as an oxidizing agent. The vanadate oxidase method has an excellent correlation with the diazo method [12]. Due to simpler and faster process than the diazo method, the vanadate method may be used alternatively for the samples containing interfering substances. Recently, an electronic instrument was designed for bilirubin measurement through the skin using vanadic acid based photometric method [13]. The method



Scheme 1. Catabolism of heme to different forms of bilirubin.



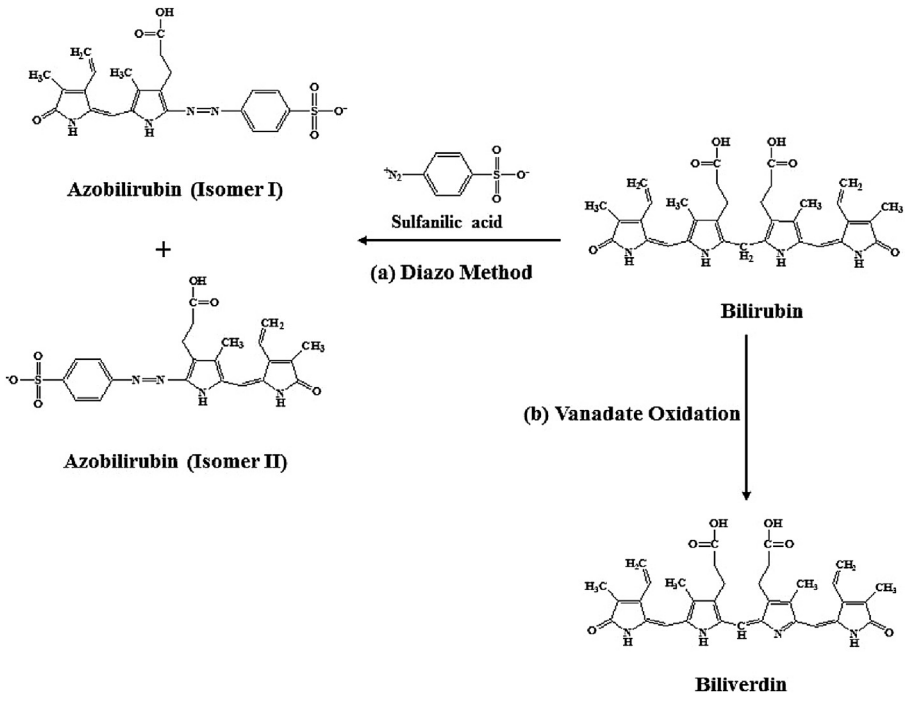
Scheme 2. Different structural forms of (a) unconjugated and (b) conjugated bilirubin molecules.

utilized two specific wavelengths to assess the bilirubin value non-invasively. The whole oxidation is completed within three minutes of measurements.

3. Advanced methods for bilirubin estimation

3.1. Chromatographic methods

Various chromatographic methods were explored to determine bilirubin sensitively [14,15]. Unconjugated bilirubin (UCB) possesses potent cytoprotective and antioxidant properties. However, it causes apoptosis and cytotoxicity when present in elevated concentration. Thus accurate measurement of UCB in body fluids, cells, and tissues is important to assess its effect on metabolism and regulations of cells. Zelenka et al. developed a highly sensitive HPLC (High Performance Liquid Chromatography)-based method to determine UCB [16]. The method determines UCB and total bilirubin both in tissue and body fluids following simple and rapid sample preparation steps. In the recent past, a new method was developed for direct determination of bilirubin in serum [17].



Scheme 3. Conventional chemical method for bilirubin determination: (a) Diazo method and (b) Vanadate oxidation method.

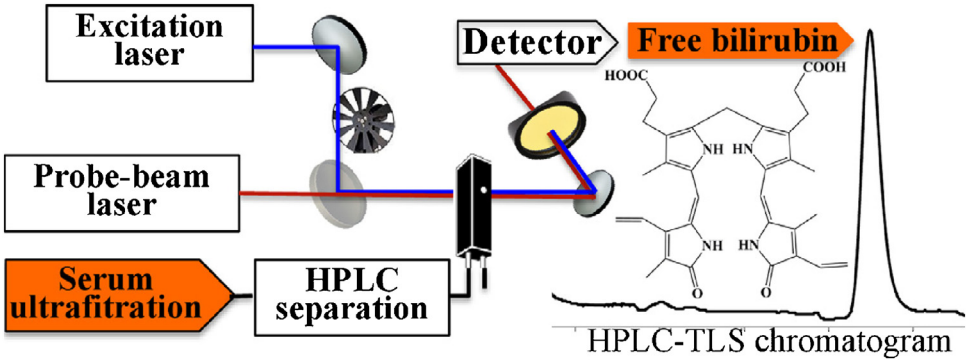


Fig. 1. Schematic representation of the direct estimation of free bilirubin in serum using HPLC-TLS coupled chromatography [17].

HPLC coupled with ultrasensitive thermal lens spectrometric (TLS) (HPLC-TLS) method was used to determine free bilirubin without involving any pretreatment or dilution of blood serum sample (Fig. 1).

Simultaneous estimation of biliverdin and bilirubin provides critical information to undertake preventive measure against some pathological conditions. An ultra-sensitive analytical method was developed for their simultaneous measurement, which involved separation of these two compounds within 11 min using isocratic reversed-phase HPLC and TLS determination [18]. The method also revealed the concentration ratio of bilirubin and unbound biliverdin in serum samples. In an another study, characterization of the kinetics of bilirubin and its multiple glucuronides was undertaken through a specific and sensitive HPLC method [19]. The method provides a vital reference for *in vivo* bilirubin metabolism, which further extended its application potential in preventive measures of bilirubin and bilirubin-related malady. It is important to understand the bilirubin end products (BOXes) because these are found in the cerebrospinal fluid after stroke and also present in heme degradation. The end products, especially the regio-isomers Z-BOX A and Z-BOX B in human serum were studied and validated with the help of liquid chromatography coupled tandem mass spectrometry (LC-ESI-MS/MS) [20]. This validation helped to provide a basis to ascertain the specific role of regio-isomeric forms of bilirubin end products in metabolism and illness.

3.2. Electrophoresis methods

Capillary electrophoresis (CE) could be employed to determine bilirubin in new borne as it is an efficient separation technique requires only a small sample volume to process swiftly [21]. Both the free- and albumin bound bilirubin were determined by using this equipment in a single phase and the work was extended to investigate drugs interacting with bilirubin-albumin complex [22]. CE coupled with fontal analysis (FA) was also reported to determine free bilirubin in mixture and its albumin binding capacity [23]. It was also found that bilirubin could be separated by adding aspirin from bilirubin-albumin mixture. The method may be employed to identify the 'residual binding capacity' to assess the hyperbilirubinemic neonates and hence, providing a scope for the risk management of bilirubin neurotoxicity in neonates. The application of CE/FA techniques was further investigated for bedside measurement of bilirubin, free bilirubin and its binding capacity with albumin through microfluidic chip [24,25]. Here, PMMA (poly-

methylmethacrylate) microfluidic chip was designed and fabricate with multi-channels to study the interaction of bilirubin with HSA. In one of these studies, ferrofluid-driven micro-mixer coupled with PMMA microchip-CE/FA was used to determine residual binding capacity and the free bilirubin. The developed method was further endorsed for clinical requirement to measure the early rising level of bilirubin in neonatal jaundice.

3.3. Electrochemical methods

Electrochemical methods, especially amperometry have been widely utilized in bilirubin sensing. Three primary approaches were involved in the determination; two of them are non-enzymatic methods, which include direct oxidation of bilirubin at the electrode surface and the use of molecular imprinting polymer for the bilirubin determination. While, the third is an enzymatic biosensing method, which employs enzyme bilirubin oxidase (Box) for the electrochemical determination of bilirubin.

3.3.1. Enzyme based methods

Box is widely used as biorecognition molecule in the electrochemical determination of bilirubin. In majority of the cases, H_2O_2 generated from the Box catalyzed oxidation of bilirubin was used as redox indicator either directly or horse reddish peroxidase (HRP) based indirect reactions to generate the amperometric signal for bilirubin. A low applied potential is preferred to generate the signal as it reduces the chance of interfering signals being formed from the co-oxidation of other electro-active species present in the sample. A dual-enzyme sensor developed by co-immobilization of Box and HRP on a graphite-epoxy matrix offered a low-potential for the determination of bilirubin with a limit of detection (LOD) of 4 μM and a linear detection range up to 10 mM [26]. Application of electron transfer mediators (ETM) facilitates to reduce the overpotential and enhances the sensitivity of the signals. Various ETMs were used with Box in different electrode configurations that generated current response linearly with the concentration of bilirubin. In one such construct, ferrocene carboxylic acid as ETM was exploited that offered shelf-life of the sensor more than three months when stored at 4 °C [27]. An amperometric biosensor fabricated with sol-gel network over a gold electrode and $[Fe(CN)6]^{-3/-4}$ as ETM for the Box immobilized on gold nanoparticles (AuNPs) was reported. The biosensor offered high sensitivity (LOD of 1.4 nM of bilirubin). However, its application potential is unclear due to its low stability [28]. In another report, a platinum electrode modified with a cross-linked adducts of Box, BSA, and glutaraldehyde was constructed. The concentration of bilirubin was determined by monitoring the H_2O_2 concentration [29]. Indirect determination of bilirubin by monitoring depletion in O_2 concentration upon enzymatic oxidation of bilirubin was also reported [30]. In their system, Box was chemically linked by using BSA and glutaraldehyde to a pre-activated membrane. The O_2 depletion was recorded using an oxygen electrode. The method was free from interference, fast and the modified electrode retained 80% of its initial activity following 30 successive measurements. Different nanoparticles were also used in the fabrication of Box based amperometric biosensors to improve the performance. Highly conductive nanoparticles are known to offer high enzyme loading on the electrode surface and facilitate to channelize electrons between the electrode and redox centre of the enzyme through direct electron transfer (DET) mechanism [31]. Feng et al. developed a nanocomposite electrode made of MWCNT, graphene and *in-situ* synthesized AuNPs over which Box was immobilized by adsorption [32]. This device exhibited a linear range of 1.33 mM–71.56 mM and an LOD of 0.34 mM for bilirubin. Subsequently, Batra et al. elaborated a composite electrode comprising of Box covalently immobilized onto zirconia coated silica nanoparticles with an LOD of 0.1 nM. The amperometric bilirubin biosensor so developed exhibited an excellent sensitivity and could be used 150 times over a period of 120 days [5]. This was further tested for determination of bilirubin both in healthy persons and patients suffering from jaundice. Here, the SiO_2 with ZrONPs as ETM helped in enhancing the performance of the constructed biosensor. An electrochemical enzyme sensor based on the synergistic catalysis of Box and graphene oxide nanoparticles on polypyrrole (Ppy) nanostructures was developed that improved LOD (0.1 nM), working range (0.01 and 500 mM), response time (within 2 s) and storage stability (150 days) without any interference [33]. In one of such reports, ascorbic acid oxidase instead of Box was used as biorecognition element for the electrochemical determination of bilirubin [34]. One of the major concerns related to biosensor development is the low stability. To curb the issue, a catalase biosensor coupled with ZnS nanoparticles was developed that determined bilirubin in the range of 3–50 μM. The sensor was not affected by some of the potential interfering agents on sensing bilirubin electrochemically [35].

3.3.2. Non-enzymatic methods

There is a plethora of reports on the non-enzymatic electrochemical methods for bilirubin determination. Several electrochemical studies were performed both in aqueous [36] as well as in non-aqueous media [37], which confirmed the electroactive nature of bilirubin. These studies further confirmed that the electrochemical oxidation of bilirubin is a multistep process in which, it is electrochemically oxidized to biliverdin, purpurin or choletelin depending upon the magnitude of the applied potential. Electrochemical measurement of bilirubin is a promising method for assessing unbound or free bilirubin in blood serum as albumin-bound fraction of bilirubin is inert for oxidation at the electrode surface [38]. Non-enzymatic electrochemical methods involving oxidation of bilirubin are mostly studied at the nanoparticle and nanocluster interface and molecular imprint platforms. In one such study, the surface of the ITO electrode was decorated with a thin layer of nickel nanoparticles. The biosensor retained 80% of its initial activity after repeated measurement of ~100 times over a period of 180 days. The results of real sample from jaundice patients and healthy persons were well correlated with the standard colorimetric methods [39]. Various other non-enzymatic electrochemical bilirubin sensors using carbon based nanomaterials such as MWCNTs [40], nanographite [41], and metallic nanomaterials such as gold nanoparticles (AuNPs) [32], gold nanorods [42] etc. were employed following different electrode fabrication techniques. Feng et al. employed *in situ* grown AuNPs onto the surface of the MWCNTs-COOH for rapid measurement of bilirubin electrochemically. A sensor with a mixed materials of ferrocene carboxide, AuNPs and MWCNTs with remarkable enhancement in the current response for bilirubin, long-term stability and good reproducibility was also reported [43]. The bilirubin sensors fabricated with MWCNTs offered high sensitivity with a response time within 5 s [40] and an LOD below the minimum critical concentration for newborns at physiological pH range [44]. Whereas, the electrochemical sensor developed by using nanographite modify platinum microelectrode could surpass the effect of interfering agents due to distinct oxidation peaks in voltammogram. The developed sensor looked promising because of its high sensitivity, stability and wide dynamic range [41]. From most of the above studies, the biocompatibility of many nanomaterials and their fast electron transfer capability were validated. An interesting proof-of-concept to determine bilirubin was developed recently in the author's lab utilizing human serum albumin (HSA) stabilized gold nanoclusters (AuNCs) as biorecognition system [45]. Here, AuNCs acted as an electron transfer bridge between bilirubin adsorbed on its natural carrier protein HSA and ITO electrode (Fig. 2). The sensor, which exhib-

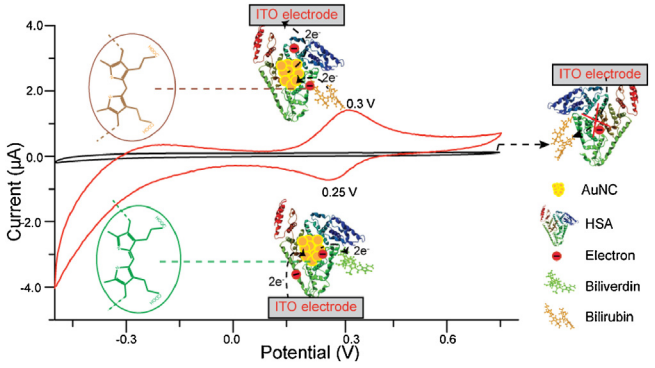


Fig. 2. Cyclic voltammetry response of HSA-AuNC modified ITO electrode for bilirubin [45].

ited high sensitivity (0.34 μA μM^{-1}) with a LOD of 86.32 nM, was applied to measure the bilirubin content in serum samples.

Bilirubin interacting with europium ions was studied with mercury electrode using electrochemical methods like cyclic voltammetry and chronocoulometry. Interestingly, bilirubin was strongly absorbed on to the mercury electrode [46]. The sensors displayed a linear range up to 300 mM and a LOD of 0.7 mM.

3.4. Spectroscopic methods

Different optical methods widely explored to determine bilirubin are presented in the following sections.

3.4.1. Fluorescence spectroscopy

The fluorescence quantum yield of bilirubin in aqueous solution is very low. However, when bound to its natural carrier albumin, the quantum efficiency of bilirubin in aqueous solution is increased that makes fluorescence measurements useful for the measurement of the bound fraction of bilirubin. The bilirubin-albumin complex emits fluorescence at $\lambda_{520\,nm}$ [47]. Based on this fluorometric measurement of albumin-bound bilirubin, the technique for determining the binding capacity was developed. The custom designed hemato-fluorometer was constructed that makes use of automated front face fluorescence measurements of whole blood for rapid micro-assay of bilirubin. The measurement of unconjugated serum bilirubin concentrations using a novel and sensitive method based on static fluorescence quenching of dansylated BSA was also developed, which required very low sample volume and determined bilirubin over a wide range of 17–343 μM [48].

An enzyme-based fibre-optic fluorescence biosensor was developed for the determination of bilirubin [49]. Later on, a miniaturized fibre optic biosensor was developed for rapid estimation of bilirubin through an indirect approach exploiting fluorescence quenching of tris(4,7-diphenyl-1,10-phenanthroline) ruthenium chloride [$Ru(dpp)_3$] caused by the dissolved molecular oxygen. In the reaction, oxygen is consumed in the oxidation of bilirubin to biliverdin, catalyzed by Box [50]. This sensor offered an LOD of 0.1 μM and a linear range of 0.1–300 μM. In another approach, inorganic metals and conjugates were also incorporated for determination of bilirubin as fluorescent dye. The fluorescent reaction of bilirubin with Zn^{2+} in dimethyl sulfoxide (DMSO) was successfully employed for the determination of bilirubin in serum. The method offers a linear range of 1.7–85 μM with an LOD of 85 nM [51]. The measurement of changes in fluorescence caused by Box catalyzed reaction was also employed for the determination of all the fractions of bilirubin following a multivariate analysis approach. The method offered a linear range upto 20 μM [52,53]. Using oxytetracycline-Eu^{3+}, tetracycline-Eu^{3+} and enoxacin-terbium as a fluorescent probe, a new spectrofluorimetric method was developed to determine even trace amount of bilirubin in serum sample [54–56]. The method is reported to be free from interference from the coexisting substances, simple and practical. This fluorescent probe remarkably enhanced the determination capability even with a trace amount of bilirubin in serum samples. The authors did a comparative analysis with the modified Jendrassik-Grof method in clinical analysis. Besides, with yttrium (Y^{3+})-norfloxacin (NLFX) complex as fluorescent probe, an easy and sensitive spectrofluorimetric method for bilirubin determination was developed [57]. This method had offered high sensitivity by simple instrumentation to determine bilirubin at low concentration. The method showed a good linear relationship in the range of 0.05–3.9 mM. Huber et al. developed a fluorescence-based sensor utilizing mutated fatty acid binding protein tagged with fluorescence dye acrylodan that specifically binds to unconjugated bilirubin [58]. The assay was based on the fluorescence quenching of the dye occurred upon specific binding of the protein with bilirubin. In addition to the quantitative measurement, study of bilirubin metabolism could also offer assessments useful to prevent many major bilirubin related health problems. The microscopic distribution of bilirubin was studied by a newly developed method on the basis of infrared femtosecond Cr:forsterite laser [59]. With this system of bilirubin molecular imaging, diagnosing of cancers or the dynamics of bilirubin metabolisms in live cells could be studied. One of the interesting features was that by using femtosecond pulses, the two photon red fluorescence of bilirubin dimers could be observed at 660 nm while the autofluorescence from other endogenous fluorophores was suppressed.

In the recent past, water soluble polyfluorene with glucuronic acid appendages was utilized for sensitive determination of free bilirubin [60]. Even though simple polyfluorenes exhibited complementary photophysical characteristics to bilirubin, the efficiency for sensing was low. Thus, the authors designed bulky pentadecyl phenol units in such a way that the modified would be self-assembled to a desired structure for the bilirubin interaction. Thus the tailor-made polyfluorenes with the synergistic effect and enhancement of bilirubin emission made this approach adaptable for visual fluorimetric color change (blue to green) based sensors. In another development, the authors continue to present new polyfluorenes with D-glucuronic acid. The role of glucuronic acid was to inhibit the adherence of the serum protein, which otherwise interferes with the determination of bilirubin [61]. The effect of serum proteins could also be neutralized by layering a polymer film containing an optimized amount of HSA over silver nano-island. The polymer layer impedes serum proteins, allows only free bilirubin to diffuse through it, which later binds to HSA in the film. Silver present below the layer enhanced the fluorescence of the HSA bound bilirubin complex up to 103 times through the mechanism of metal-enhanced fluorescence signal. Recently, a simple fluorescent platform was developed using (2,2′-((1E,1′E)-((6-bromopyridine-2,3-diyl) bis(azanylylidinediphenol) (BAMD) [62]. After the imine compound was synthesized by a green and facile approach, the platform was used to determine bilirubin in biofluid using both colorimetric and fluorometric techniques in physiological and basic pH values. BAMD had shown wonderful fluorescent intensity and under optimized condition, the sensor selectively determined bilirubin in the presence of other biomolecules and metal ions. To increase the sensitivity and speed of the assay, metallic surfaces were used to enhance intensity of fluorescence species or reactions. An invention related to metallic-surface determination system was reported. The metal-enhanced fluorescence technique was applied for the specific assay of free bilirubin in neonatal serum [63]. In one of the rare study, instead of using any chemical fluorescent compound, a fluorescent protein was used as probe. UnaG, a fluorescent protein from Japanese eel muscle that specifically binds with unconjugated bilirubin was exploited to determine

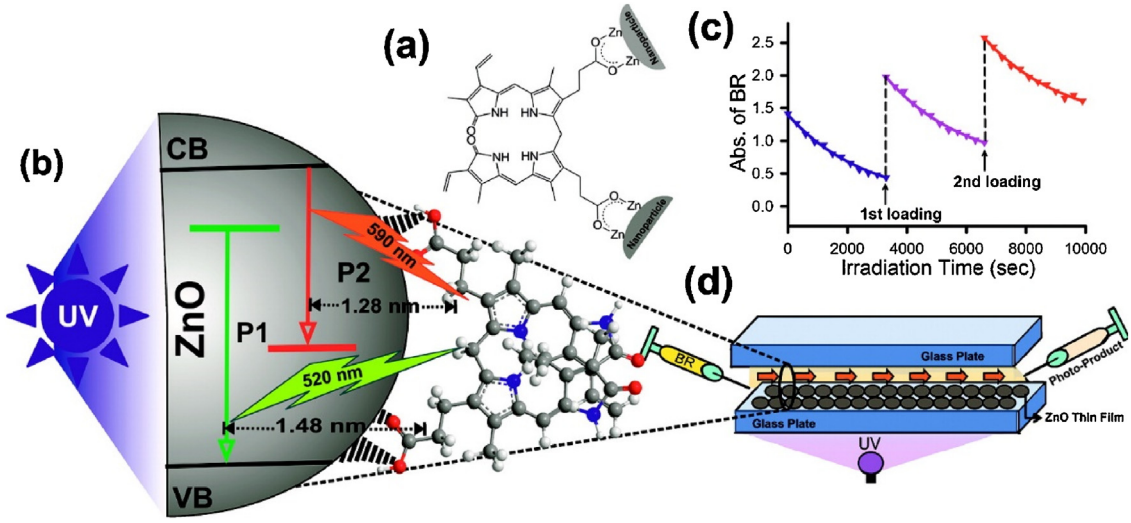


Fig. 3. Photodegradation of bilirubin using ZnO NPs as phototherapeutic agent (a) Molecular structure of bilirubin on the surface of ZnO NPs. (b) FRET dynamics showing different oxygen vacancy centers. (c) Photocatalytic degradation curves (abs. vs t) of bilirubin in ZnO suspension under UV-light irradiation for three repeated cycles. (d) A schematic representation of the designed flow-device using ZnO NPs as phototherapeutic agents. [67].

the levels of unconjugated bilirubin in serum [64] and to assay biliverdin reductase activity [65]. The UnaG based method could determine bilirubin concentration as low as 1 nM. In this method, the extraction of bilirubin before measurement was not required and repeated measurements of bilirubin production from the same sample could be monitored. Recently, a newly designed fluorescent metal organic framework (MOF) was used for bilirubin sensing [66]. This system could monitor free bilirubin in human serum sample bearing potential for clinical applications to diagnose jaundice. To understand the mechanism of bilirubin degradation, recently, a novel non-radiative energy transfer pathway was reported for ZnO nanoparticles (NPs) [67]. In order to enhance the efficiency of the bilirubin photodegradation process, the authors demonstrated the potential use of ZnO NPs as phototherapeutic agents which showed a strong optical absorption in the near UV region as shown in the Fig. 3. About 6% and 51% of the total bilirubin was degraded in the absence and presence of ZnO NPs, respectively under the same experimental condition.

Santhosh et al. reported a sensitive and reliable method for bilirubin determination using HSA stabilized AuNCs as fluorescent probe. The fluorescence of the AuNCs was strongly quenched by bilirubin in a concentration dependent manner [68]. The fluorescence response was practically unchanged over a wide pH (6–9) and temperature (25–50 °C) range. Additionally, the peroxidase-like catalytic activity of these nanoclusters was also exploited for colorimetric estimation of bilirubin in serum sample.

3.4.2. Spectrophotometry

Bilirubin could be measured by different spectrophotometry methods among which the Van den Bergh reaction based method is well acclaimed. The method involves a reaction of bilirubin with sulfanilic acid giving a red azodipyrrole. To reduce the interference involved in the method caused by the substances in serum and in urine, various attempts were made. In one such attempt, the analytical procedure was established in presence of the biocatalytic system hemoglobin/glucose oxidase/glucose. Notably, both hemoglobin and myoglobin show pseudocatalytic activity when combined with H_2O_2. In this, polymeric support was obtained from poly(vinyl alcohol) or which has high biocompatibility. Polyhemoglobin from the same hemoglobin was also used as biochemical reagents allowing an *in vivo* monitoring of bilirubin. The exploitation of hemoglobin as catalyst offered an inexpensive and sensitive system [69]. One of the most frequently performed tests for serum bilirubin in newborns is the direct spectrophotometric method [70]. It involves the measurement of absorbance at two wavelengths 454 and 540 nm. Here, bilirubin absorbs at 454 nm, whereas, hemoglobin absorbs equally at both 454 and 528 nm. Hence, subtraction of the absorbance at 528 nm from that at 454 nm yields a value that can be attributed largely to bilirubin. However, the method was inaccurate due to the prevalence of the other forms of bilirubin and chromophores in older children and adults that limits the use of this technique to neonates with age below 2–3 weeks. A new total bilirubin determination method based on multiple wavelength absorbance measurements and an algorithm to calculate concentration were evaluated [71]. The method thus overcame the limitations involved in earlier spectroscopic methods providing reliable results. One of the main purposes of the study was to evaluate the accuracy and precision of the developed method by radiometer in conjunction with blood gas analysis/co-oximetry (ABL 735).

The automation of bilirubin determination was also introduced. However, no flow-injection (FI) methods were used to determine bilirubin until Fernandez-Romero et al. introduced it for the first time in 1993. They proposed the bilirubin determination by two automatic spectrophotometric methods based on the utilizing flow-injection analysis [72]. Total, direct and indirect bilirubins were determined following two flow injection methods performing simultaneously. In another development of full automation, different group for the determination of urobilinogen and bilirubin developed simultaneous injection effective mixing flow analysis (SIEMA) system. The developed system is consisting of a syringe pump, connectors, holding coils, mixing coils, and spectrometer [73]. In this 4-channel SIEMA system, two channels were used for the measurement of urobilirubin and the other two were used for bilirubin determination. The developed system exhibited the LODs for urobilinogen and bilirubin of 1.0 mg L^{-1} and 0.003 mgL^{-1}, respectively. The authors suggested that the proposed methods could be applied to determine trace amount of urobilirubin and bilirubin in urine samples. The developed SIEMA system was also successfully used to determine bilirubin and creatinine in urine sample [74]. In this, the LODs of bilirubin and creatinine were found to be 7 μg L^{-1} and 0.6 mg L^{-1}, respectively.

3.4.3. Luminescence spectroscopy

Chemiluminescence (CL) is a highly sensitive technique with great potential for routine usage in clinical laboratories. It has been intensively used for bilirubin determination. Wu et al. reported first that bilirubin in an organic solvent emits light because of peroxy-oxalate chemiluminescence reaction. The relative and absolute LOD were found to be 8.5 nmol L^{-1} and 5.0 ng, respectively. The bilirubin CL could be determined in N,N'-dimethyl formamide (DMF) where the reaction of bis(2,4,6-trichlorophenyl) oxalate and hydrogen peroxide could be possible but not in DMSO, chloroform, or pyridine. Later on, Palilis et al. developed a chemiluminogenic redox reaction of bilirubin in aqueous media. The reaction of bilirubin with N-bromosuccinimide was investigated using flow injection analyzer and with hypochlorite further analysis was performed using a continuous flow analyzer [75,76]. With this method, bilirubin could be determined reproducibly down to the level of 1 ng mL^{-1}. However, this method encountered interference from albumin and low selectivity, which hampered its application in real sample analysis. To enhance the CL efficiency, micellar media was incorporated to study bilirubin and its conjugates [77]. In this method, a very strong oxidant, peroxynitrite ($ONOO^-$) generated from peroxynitrous acid (ONOOH) in NaOH solution as a result of the reaction of nitrite with acidified H_2O_2. Bilirubin and its conjugate, in a suitable micellar solution, enhanced the CL emission remarkably from nitrite-H_2O_2 reaction which offers to measure total bilirubin contents in serum. The usefulness of the method was demonstrated from its selectivity, precision, and recovery studies. Since CL is generated usually by fast reactions, there could be imprecise measurements due to the irreproducible mixing of sample and reagents. So, a new CL method combined with flow injection analysis technique was introduced which was based on the inhibition effect of bilirubin on CL from the lucigenin-H_2O_2 system in an alkaline medium [78]. The decrease in CL intensity was proportional to the concentration of bilirubin in the range 0.1–100 μM. The LOD discerned from the developed calibration plot was ~13.5 nM. On the same occasion, the previously mentioned work of Lu et al. was extended to enhance the sample pre-concentration by on-line incorporation of cloud point extraction (CPE) to flow injection analysis (FIA) for the determination of total serum bilirubin [79]. A new nanotechnology-based luminescent sensor for hyperbilirubinemia was reported [80]. The chitosan-stabilised AuNCs embedded in polyvinylidenedifluoride (PVDF) membrane was used to determine bilirubin with high sensitivity. A new chemiluminescence method was proposed to quantify bilirubin which combined FIA technique and on the inhibition effect of bilirubin from the lucigenin-H_2O_2 system. This interference-free, rapid and simple method could determine as low as 7.8826 ng mL^{-1} of bilirubin.

Bioluminescent sensors for bilirubin determination using multifunctional nano-sized materials is another development in the recent past [81]. In this work, magnetic silica particles were modified using guanidine containing co-polymers which were manufactured using sol-gel method. The surface of the polymer was further functionalized with bilirubin-inducible fluorescent protein UnaG. In an aqueous bilirubin media, bright fluorescence of silica particles decorated with polymer-UnaG was detected and used the phenomenon to determine bilirubin.

3.4.4. Evanescent waves or optical fiber

Fiber optical sensor offers several advantages such as, the reference signal may be avoided and electrical and chemical interference could also be eliminated. A fiber optic sensor based on fluorescence quenching by oxygen consumption was developed for quantitative estimation of bilirubin [49]. This Box based enzymatic oxidation reaction depletes the level of molecular oxygen in the sample thus recovering the fluorescence emission of ruthenium complex from the quenching effect of oxygen. The method was further improved by immobilizing both enzyme and fluorescence indicator in an acrylamide polymeric support through controlled photopolymerization [50]. The LOD of the sensor was 10 μM, the linear dynamic range was 10 μM to 30 mM and the response time was 10 s presenting 50-fold improvement over the existing sensor.

Fiber optic consists of inner core and an outer cladding. A new sensor was developed where an unclad region was created to allow the evanescent of the transmitted light to interact with the environment. Conjugated and unconjugated bilirubin showed different response and wavelength signal thus offering a scope to measure both the bilirubins by two optical frequencies. The sensor could be employed for real-time monitoring and point-of-care examination of neonates with hyperbilirubinemia [82]. In conventional treatment of neonatal jaundice phototherapy is done and bilirubin level is monitored time to time following blood test. A non-invasive method to monitor the bilirubin level was proposed [83], where evanescent field based optical fiber strategy that is coupled to the aqueous environment was used to continuously monitor the degradation of bilirubin level.

3.5. Molecular imprinting (MIP) techniques

The MIP is usually synthesized by using functional and cross-linking monomers through co-polymerization process in the presence of the target analyte [84]. When the polymerization process is completed, the template molecules are extracted from the polymer matrix that creates highly specific cavities, which is then used as molecular template. The templates are complimentary in chemical functionality, shape, and size to the analyte and can trap the target molecules into the cavities. MIP offers stability even in a wide range of environmental conditions. It is cheap, easy to synthesis, and selective thus exhibits wide application potential [85,86]. MI techniques were used for the quantitative measurement of bilirubin considering the fact that the conventional Box based enzymatic method encounters many limitation and challenges as the oxidase loses activity under various environmental conditions. A molecular template for α-Bilirubin was designed by polymerization of methacrylic acid (MAA) with an initiator 2,2-azobisisobuthyronitrile (AIBN) and agent ethylene glycol dimethyl acrylate (EGDMA) and successfully produced poly(methacrylic acid-co-ethylene glycol dimethylacrylate) (poly(MAA-EGDMA) for the bilirubin determination [87]. Since this development, parameters like sensitivity and selectivity, ionic effect, allosteric binding of bilirubin, biliverdin, bilirubin/biliverdin mixtures were analyzed [88–90]. β-Cyclodextrin (β-CD) as functional monomer was explored for the determination of bilirubin [91]. The specific recognition ability of the developed imprint was attributed to the cooperative effect and hydrogen bonding. A linear range of 0.1- 0.8 mM and an LOD 0.08 mM, respectively for bilirubin were discerned with the developed sensor. In another development, a molecular imprinted super macroporous cryogel was used to recognize bilirubin [92]. N-methacryloyl-(L)-tyrosinenethyl ester (MAT) was used as the organizational monomer to exploit bilirubin measurement. Advantages like large pores, short diffusion path, and low pressure drop and very short residence time of cryogels were explored for the adsorption of bilirubin from blood serum. With this bilirubin imprinted poly(hydroxyethyl methacrylate-MAT [poly(HEMA-MAT)] bilirubin-MIP cryogel, selective bilirubin adsorption was accomplished and additionally, adsorption with testosterone and cholesterol was also studied as competitive substances. In another development, a fluorescent imprinted polymer that binds bilirubin selectively was developed. The imprinted polymer was synthesized by using functional monomer-methacrylic acid and fluorescent zinc(II) protoporphyrin. The bilirubin imprinted poly(zinc protoporphyrin-methacrylic acid-ethyl glycol dimethylacrylate) [poly(ZnPP-MAA-EGDMA)] was

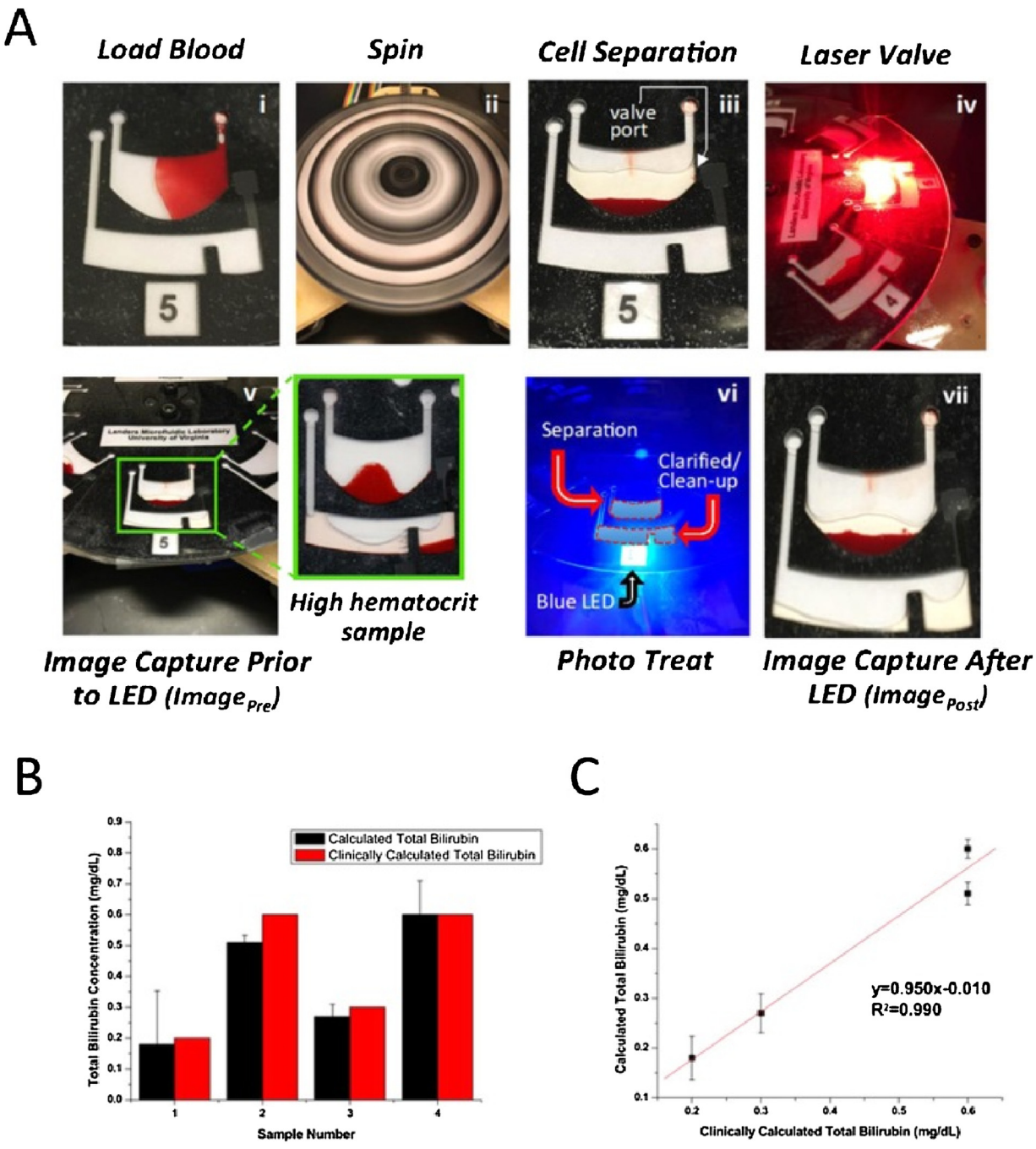


Fig. 4. BiliDisc protocol and comparison of calculated total bilirubin values versus conventional clinical laboratory methods: (A) Series of images demonstrating the BiliDisc protocol. (B) Comparison bar graph displaying the good agreement between total bilirubin values obtained with conventional methods and the microfluidic bilirubin assay. (C) Deming-regression plot showcasing the clinical accuracy of the BiliDisc protocol for four different blood samples [99].

used to imprint with bilirubin which could cause spectroscopic change because of the metal-ion coordination [93]. This interesting fluorescent imprinted polymer could selectively bind to α-bilirubin.

3.6. Piezoelectric techniques

One of the interesting applications of piezoelectric principle is the quartz crystal microbalance (QCM), which detect mass changes sensitively. Owing to its various advantages such as, high sensitivity, inexpensive, simple, and portability, QCM technology was used for bilirubin determination as well. MIP technique coupled with QCM was used to develop a highly selective and sensitive bilirubin determination system. This coupling technique was developed by coating bilirubin imprinted polymer on a thiol modified gold electrode of QCM chip [94]. Bilirubin imprinted polymer (BIP) was made by using monomer (4-vinylpyridine), cross-linker (divinylbenzene), and initiator (benzophenone). The BIP/QCM chip offered high selectivity and could be used for more than seven months continuously. Molecularly imprinted hydroxyapatite (HAP) film was modified to determine bilirubin through the piezoelectric method [95]. This bilirubin biosensor exhibited high sensitivity, reproducibility, short response time (37 min). In another development, titanium was used in which the molecular imprinting and surface

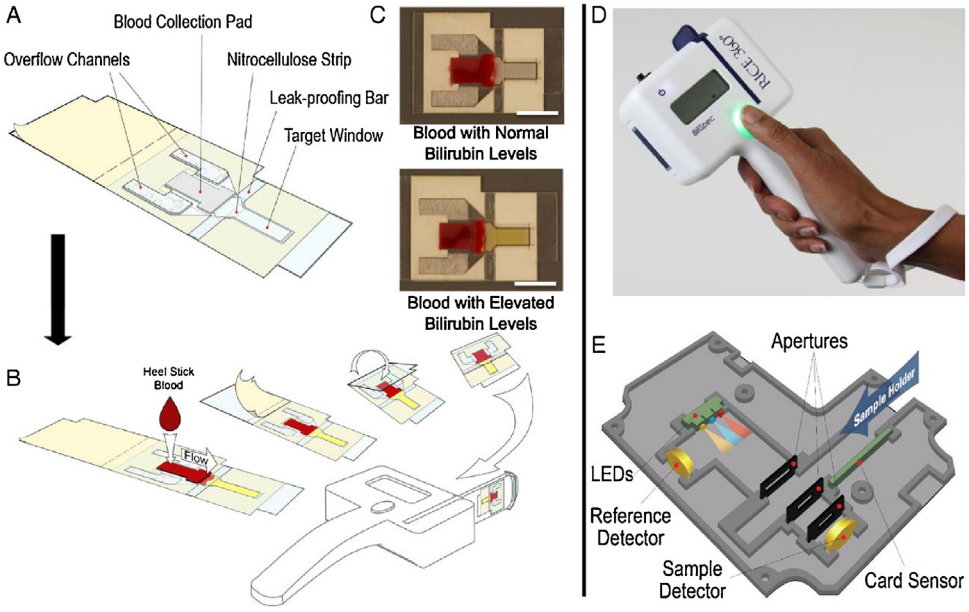


Fig. 5. BiliSpec lateral flow card and reader. (A) Diagram of the lateral flow card components. To use the card, blood is applied to the collection pad, protective paper is removed from the adhesive-coated acetate, and the card is folded to seal blood inside. (B) Plasma flows down to the distal tip of the card before being inserted into the reader for measurement. (C) Representative photographs of sealed lateral flow cards following application of blood with normal and elevated TSB levels. (Scale bar, 1 cm.) (D) Photograph of the hand-held reader. (E) Diagram illustrating the internal optical and electronic layout of the reader [100].

sol-gel methods were used for casting bilirubin-imprinted titania film onto a quartz crystal sensor [96]. The method exhibited high sensitivity, good reproducibility, short response time, wide linear range of detection and low LOD. The method was also validated in serum samples. By MIP technique, bilirubin-imprinted polymer Poly-(2-hydroxyethyl methacrylate-*N*-methacryloyl-L-tryptophan methyl ester) (PHEMATrp) nanofilm was synthesized on a gold sensing platform of QCM [97]. The system was then used for determining bilirubin in human serum. With this method, the LOD and limit of quantification (LOQ) were calculated to be 0.45 μg mL^{-1} and 0.9 μg mL^{-1}, respectively. In this development, the authors tried to solve the mass transfer restriction problem of macromolecules across the matrix by incorporating nanosized materials. The nanofilm of bilirubin-imprinted surface was achieved by polymerization under UV light.

3.7. Point-of-care testing device

Miniaturization of analytical device is a focused area in the current technological venture for point-of-care testing (POCT) applications. With the advent of advance techniques such as microfluidics, micro-electro-mechanical system (MEMS), lithography and advance materials such as, nanomaterials and polymers, the miniaturization of device with desired functionalities could be achieved. Portability, low cost, simple operation, and less sample volume are the common advantages offered by the miniaturized devices. Besides, screen printed electrodes (SPEs) functionalized with nanomaterials were used for bilirubin determination. MWCNTs and graphene on SPEs enhanced the electrocatalytic activity for bilirubin [98]. There was also some development for bed-side monitoring of free bilirubin [25]. In this study, a microfluidic chip was first fabricated to analyze free bilirubin and its interaction with HSA. The multichannel chip of poly(methyl methacrylate) (PMMA) was used for determining bilirubin with high sensitivity, selectivity and precision. Another PMMA microfluidic chip-CE device was introduced with multi-segment circular-ferrofluid-driven micromixing injector to determine bilirubin [24]. Recently, a simple, reagent-less quantification of total bilirubin based on microfluidic photo-treatment was developed [99]. Even though there has been significant development in measuring serum bilirubin and treatment thereof for the jaundice neonatal, one important aspect of monitoring the total bilirubin in newborns not only during the onset of kernicterus but also following termination of the infant phototherapy is yet to be properly addressed. Thompson et al. [99] developed a microfluidic total bilirubin quantification technique using a polyester chip (Fig. 4). The approach has opened a scope to integrate inexpensive device for multiplex testing while providing rapid results.

Microfluidic devices were extended to paper-based platform where the inherent wicking property of paper answered the problem associated with automation in microfluidic devices. The simplicity of the method along with the advantages being offered by the microfluidic paper-based analytical devices (μPADs) has stimulated further research interest. Cardoso et al. designed a μPAD bearing application potentials for analytes commonly present in urine and serum sample. The developed device could determine 12.7 mg dL^{-1} of bilirubin in the sample. In a parallel development, a POC device for low-resource settings was developed with a low-cost reader and disposable lateral flow card [100]. The performance of the device (BiliSpec) was checked with real samples obtained from the hospital and the results were compared with the standard laboratory reference (Fig. 5).

The total serum bilirubin (TSB) measurement following chemical methods to assess hyperbilirubinemia in neonates as discuss elsewhere is remain gold standard. However, these invasive, painful, and expensive methods are gradually replaced by transcutaneous bilirubinometer [101]. Transcutaneous bilirubinometry (TcB) is now widely used in birth centers and in the outpatient department because of its (a) simple operation, (b) non-invasive nature, (c) less expensive, and (d) rapid detection capability. Some of the currently available TcB devices are (a) Drager jaundice meter (JM-103™), (b) BiliChek™, (c) BiliTest GB77L™, (d) BiliStick, (e) BiliMed™. However, TcB method is still a screening test and hence, before initiation of the therapy the result should be con-

Table 1
The various prominent analytical methods used for bilirubin measurements.

Technique	LOD	Linear range	Reference
	–	3.1–348 mg L^{-1}	[106]
	10 pmol $UCBg^{-1}$	–	[16]
	90 pM	0.250–150 nM	[17]
Chromatography	0.5 nM	–	[18]
	450 nM	0.01–2 μM	[19]
	14.4 ± 5.1 nM (Z-BOX A)	2.74–163 pgμL^{-1} (Z-BOX A) 2.12–162.4 pgμL^{-1} (Z-BOX B)	[20]
	10.9 ± 3.1 nM (Z-BOX B)		
	0.24 μg L^{-1}	1.0–40.0 μg L^{-1}	[107]
	2 μM	5–206 μM	[22]
Electrophoresis	30 and 150 nM	–	[108]
	9 μM	10–200 μM	[25]
	0.7 μM	300 μM	[29]
	8×10^{-6} M	2.0×10^{-4} M	[30]
	0.15 μM	0.01–600 μM	[39]
	0.014 μM	0.8 μM–10 μM	[40]
	4.2 ± 0.1 μM	–	[44]
	56 μM	100–500 μM $μA^{-1}$	[41]
	40 ± 3.8 nM	0.1 μM and 50 μM	[34]
Electrochemical	1.4 nM	1–5000 μM	[28]
	0.1 nM	0.01–500μM	[33]
	25.0 ± 1.8 nM	1.2 μM–0.42 mM	[109]
	4.0×10^{-7} M	1.0×10^{-6} and 1.0×10^{-3} M	[110]
	0.12 μM	1–100 μM$μA^{-1}$	[43]
	0.34 μM	1,33–71.56 μM	[32]
	0.1 nM	0.02–250 mM	[5]
	30 pM	100 nM–1pM	[111]
	0.05 μM	0.1–50 μM	[96]
Piezoelectric	0.01 μM	0.05–80 μM	[112]
	0.2 μM	–	[52]
	0.04 μM	0.1–50 μM	[93]
	0.77 μM	0.5–30 μM	[55]
	0.15 μM	25–50 μM	[61]
	2.8 ng mL^{-1}	0.03–2.3 mg mL^{-1}	[57]
	8.1×10^{-8} M	1.0×10^{-7}-4.5×10^{-6} M	[56]
	5 μg dL^{-1}	0.1–5 mg dL^{-1}	[51]
	2.8pM	1 pM–500 μM	[62]
	–	Enzymatic method: 0.09–25.0 μM (total bilirubin) 0.14–13.0 μM (direct bilirubin) Non-enzymatic method: 0.85–144.0 μM (total bilirubin) 3.80–125.0 μM (direct bilirubin)	[72]
Optical			
	50 ng mL^{-1}	0.2–20 μg mL^{-1}	[75]
	7.8826 ngmL^{-1}	0.0585–58.47 μg mL^{-1}	[113]
	2.60 μg mL^{-1}	3.30–8.60 μg mL^{-1}	[114]
	–	10–200 mmol	[25]
	5.0 μM	–	[115]
	7 μgL^{-1}	240–500 μg L^{-1}	[74]
	10 ng mL^{-1} (bilirubin) 8 ng mL^{-1} (bilirubin conjugate)	–	[77]
	1.8 μg L^{-1}	5–120 μg L^{-1}	[79]
	0.068 μM	0.068–17.2 μM	[116]
	1.0 mg L^{-1} (urobilinogen) 0.003 mgL^{-1} (bilirubin)	Upto 100.0 mg L^{-1} (urobilinogen) Upto 5.0 mg L^{-1} (bilirubin)	[73]
	–	Upto 7 mg L^{-1}	[52]
	0.007 μM.	0.1– 17 μM	[117]

firmed by TSB method. Moreover, the correlation between TSB and TcB is affected by various factors like race, gestational age, birth weight, skin color, and types of TcB instruments [102–105]. On the other hand, there are few bilirubin diagnostic kits available which outperforms its conventional counterparts. Quantichrom™ bilirubin bioassay kit is a simple and accurate one, with an LOD of 0.16 mg dL^{-1}. The assay can be performed in 96-well plate with test duration of ∼10 min. "Total Bilirubin" of Thermos Fischer Scientific is another kit which is used for diagnostic purpose. BioVision Inc.'s bilirubin (total and direct) colorimetric assay kit is another simple robust and reliable one. Accurex Biomedical Pvt. Ltd. also produces bilirubin detection kit, Autozyme Bilirubin, a colorimetric assay method with test duration of 5 min. However, the currently available diagnostic kits have shortcomings like short shelf-life, storage conditions, expensive, require skill operator.

The critical performances (LOD and linear range) offered by some prominent bilirubin determination techniques reported under different principles are included in Table 1.

4. Conclusions and future prospects

Presently, most widely used methods for measuring the serum bilirubin levels are (a) Laboratory, (b) Capillary and (c) Transcutaneous methods. Among these, the transcutaneous method has been increasingly used for neonates due to its non-invasive nature, ability to deliver fast and reliable results useful for preliminary

diagnosis of hyperbilirubinemia. Based on the principles various diagnostic kits and devices have been manufactured and marketed by many companies useful for point of care settings, birth centers and outpatient departments. The research on the subject is however, still widespread to develop new and better methods of bilirubin determination as witnessed from the volume of publications generated from the last couple of years. Attempt has been made to develop sensitive and selective methods with broad dynamic range to detect bilirubin directly in biological samples following mostly, optical, chromatographic, electrochemical, and piezoelectric principles. There is however, an additional demand for precisely measuring the different fractions such as, conjugated, unconjugated and free forms of bilirubin and its intermediate metabolic products (biliverdin and other end products) in serum that has also intensified the research. The investigations on this front is mostly engaged on exploring various chromatography-based techniques due to the obvious reason of advantages that could be accrued through the separation processes to selectively detect and measure the targets. The bilirubin detection and monitoring systems, particularly, for severe neonatal jaundice in low and middle income countries are yet to develop properly. The introduction of inexpensive, simple and rapid detection devices, for measuring bilirubin and effectively treating severe neonatal jaundice is essential in those locations. To develop such low cost and portable device for resource limited environment and POC settings, attempts have been made, where the advance materials, techniques and concepts from multiple disciplines are explored. Among the materials and techniques, nanomaterial and micro fabrication techniques are increasingly studied following the similar trend of advance biosensor research. Incorporation of modern communication techniques and devices such as, smartphones, blue tooth and allied wireless signal transmission facility with the devices could offer mobility, portability, and applicability of the developed sensor in remote and resource limited environment. This review witnessed significant achievements in developing sensitive and highly selective analytical methods and portable techniques for bilirubin determination over the last 5 years. However, for commercial perspective, the bilirubin determination devices with multiplex capability covering other analytes of clinical importance may be suggested. Moreover, such methods and techniques should preferably operate non-invasively as the conventional invasive approaches are not suitable for newborns and growingly disregarded by the patient due to pain and infection.

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