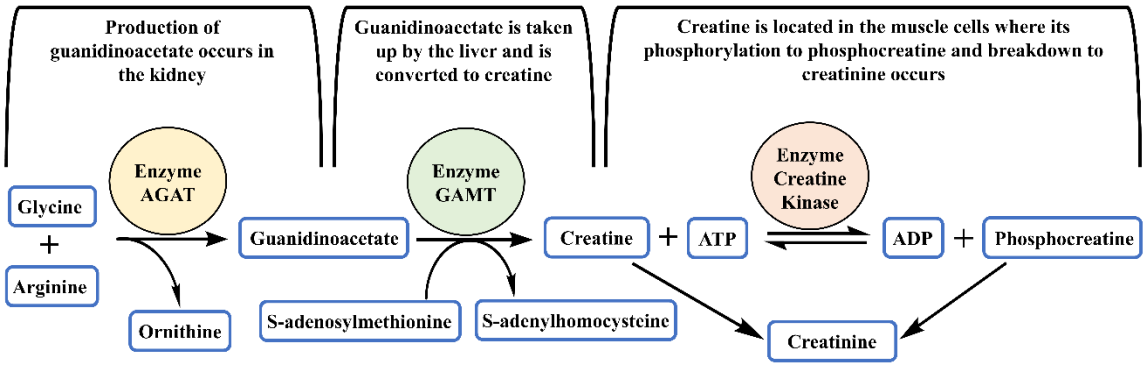
Chapter 1

A general introduction to creatinine, its importance and its detection techniques

1.1 Introduction to creatinine: a significant metabolic waste product

'Metabolic processes' and 'life forms' are two inextricable terms, as every living organism is an abode to multiple chemical synthesis (anabolism) or breakdown (catabolism) reactions that are imperative for its survival. Decades of research have been dedicated to decoding the intricated mechanistic pathways of those metabolic processes and research in this field continues. While many of the proposed mechanistic pathways have widely been accepted, the precise mechanisms for some processes are still unbeknownst. The metabolic processes yield several products and waste products, and one example of a waste product formed in the muscle tissues by the catabolism of creatine {IUPAC name: 2-[Carbamimidoyl(methyl)amino]acetic acid} is a nitrogenous base named creatinine (IUPAC name: 2-amino-3-methyl-4*H*-imidazol-5-one). It has been reported that the biosynthesis of creatine in the human body involves three amino acids, namely, arginine, glycine and methionine; two enzymes, namely, L-arginine:glycine amidinotransferase (AGAT) and glycine N-methyltransferase (GAMT); and, meanwhile, follows an inter-organ (kidney to liver) pathway [1]. Following its biosynthesis, creatine is ingested into the bloodstream and located predominantly in the skeletal muscles, which contain approximately 95% of the total creatine pool [2, 3]. The muscle cells offer the site where a reversible phosphorylation reaction of creatine occurs for energy storage to produce phosphocreatine, catalyzed by the creatine kinase enzyme [4, 5]. However, both the compounds, creatine and phosphocreatine, break down spontaneously, non-enzymatically and irreversibly in the muscle cells to produce creatinine [4, 5]; the exact cause and chemistry behind which is not yet well-established. The biosynthesis of creatine and creatinine has been illustrated in Scheme 1.1.



Scheme 1.1: Steps involved in the biosynthesis of creatine and creatinine.

Despite being a waste product of muscle metabolism which ought to be excreted out of the body via urine, creatinine holds great significance in the field of healthcare and research. After permeating the muscle cell membranes, creatinine diffuses into the bloodstream and is eventually filtered out by healthily functioning kidneys through glomerular filtration [6]. The glomerular filtration rate (GFR), which can be defined as the rate at which the filtration through the glomerulus occurs (or, the rate at which the filtered fluid flows through the kidneys), is a standard measure of renal function. When an individual suffers from Chronic Kidney Disease (CKD), several symptoms such as nausea, fatigue, pain, anxiety, insomnia, etc. may show up [7]. Simultaneously, the individual's GFR decreases and the concentration of creatinine in serum or urine consequently deviates from normalcy. The normal range of serum creatinine varies from 0.7 to 1.5 mg/dL for healthy males and from 0.6 to 1.4 mg/dL for healthy females [8], while creatinine level in normal human urine ranges from 280 mg/L to 2590 mg/L i.e., from 2.48 mM to 22.92 mM [9, 10]. Any deviation from these normal ranges would indicate a poor GFR which reflects malfunctioning of kidneys. Hence, creatinine is a direct marker for any renal dysfunctionality and the accurate determination of creatinine in body fluids is of utmost necessity to monitor the renal function of an individual.

A question that can be posed here is why creatinine, among several other biological components, is widely accepted as the marker for renal dysfunctionality. The answer lies in the catabolism rate of muscular creatine and phosphocreatine as the non-enzymatic conversion of these compounds to creatinine occurs at an almost constant rate of 2 % per day [11], which results in a fairly stable concentration of creatinine in the serum unless deviated due to renal malfunctions. Furthermore, creatinine is also filtered freely, unbound to any protein, by the glomerulus and does not get reabsorbed in the renal tubules either [12]. Historically, urea and blood urea nitrogen (BUN) have also been used as markers to assess renal function [13]. In fact, urea was the first used marker in this context, followed by BUN in the mid-1900s [13]. However, non-renal factors like diet and urea cycle enzymes can lead to the overproduction of urea, and soaring of the BUN level due to pregnancy or intake of protein-rich food has also been reported [14]. Thus, urea and BUN are regarded as poor markers, while creatinine emerges as more reliable.

Some low molecular weight proteins (LMWPs) such as β_2-microglobulin (B2M), β-trace protein (BTP) and cystatin C are also currently in focus as potential renal function

markers [13, 14]. It is debatable if the determination of B2M concentration in urine can evaluate renal functions precisely, as some studies have proven that B2M mostly gets destroyed by proximal tubular cells after it's filtered through the glomerulus, which markedly lowers its quantity in daily excreted urine to below 400 ng [15, 16]. Nevertheless, its serum level increases with a decrease in renal functions [15, 17] and, unlike creatinine, its concentration in serum is not dependent on muscle mass [18]. Therefore, recently, some researchers [19-26] have explored the usage of B2M as a renal function marker but no major advantage over creatinine can be claimed as its concentration in serum is reported to increase due to many other factors like lymphoproliferative disorders [27]. BTP exhibits properties similar to B2M as the former also gets filtered through the glomerulus in a free state and reabsorbed by the proximal tubular cells, with its concentration in serum being proportionally affected by deteriorating GFR [28]. Thus, BTP has also been utilized as a laboratory tool to assess the functioning of kidneys [24-26] but it has been critiqued that BTP shows no improvement in accuracy or precision over other renal function markers [29]. Amongst the low molecular weight proteins, cystatin C has been the most explored one in this regard [30-39]. While some studies in the early 2000s acknowledged the superiority of cystatin C over serum creatinine due to being independent of age, gender and muscle mass, and being more efficient in determining GFR or renal functions in patients suffering from diabetes (type 1 and type 2) or cirrhosis [40-44], some latest investigations have outlined the dependence of cystatin C levels in the serum on several non-GFR factors like steroid intake, thyroid dysfunction, adiposity and inflammation [45]. In comparison to creatinine, another challenge for the LMWPs in the discussion is their respective normal concentration in serum; 1.5 to 3 mg/L for B2M, < 0.70 mg/L for BTP and 0.59 to 1.04 mg/L for cystatin C [46, 47]. The normal concentrations of these LMWPs are many-fold lower than the normal concentration of serum creatinine, which presents an uphill task to the researchers as the detection techniques for the LMWPs have to be comparatively more sensitive to detect the lower concentrations precisely.

From the brief discussion above, it can be argued that although some other biological components are being considered for the development of a better renal function marker, several adversities have to be addressed and thus, the significance of creatinine as the most reliable and widely accepted renal function marker is unlikely to be obsoleted in the near future.

1.2 Creatinine clearance and GFR: formulas, comparison and ranges

Another important term that emphasizes the role of creatinine and needs to be discussed in this chapter is 'creatinine clearance' (CL_{cr}). CL_{cr} can be defined as the volume of plasma from which creatinine gets completely filtered out due to glomerular filtration in unit time. A CL_{cr} test provides an assessment of the functioning of the kidneys, much like the determination of GFR does.

$$\mathrm{CL_{cr}} = \frac{(\mathrm{UCr}) \times (\mathrm{V_U})}{\mathrm{SCr}}$$

(Eq. 1.1)

Equation 1.1 represents the traditional formula used for determining CL_{cr} (in mL/min), where UCr is the urinary creatinine concentration (in mg/100 mL), SCr is the serum creatinine concentration (in mg/100 mL) and V_U is the volume of urine collected in 24 hours [48]. This traditional formula merely compares the creatinine concentrations in serum and urine. In a report published in 1959, Edwards and Whyte [49] used another formula which highlighted the importance of body surface area (BSA, expressed in m^2) for calculating CL_{cr}, (in mL/min/1.73 m^2) as represented by Equation 1.2.

$$\mathrm{CL_{cr}} = \frac{(\mathrm{UCr}) \times (\mathrm{V_U}) \times 1.73}{\mathrm{SCr} \times \mathrm{BSA}}$$

(Eq. 1.2)

BSA can be calculated by using the Du Bois formula [50], where body weight (BW) is expressed in kg and height (Ht) in cm, as represented by Equation 1.3.

$$\mathrm{BSA} = 0.007184 \times \mathrm{BW}^{0.425} \times \mathrm{Ht}^{0.725}$$

(Eq. 1.3)

Some detailed analyses further identified the dependence of CL_{cr} on multiple factors like age, BW, BSA, Ht, creatinine production rate and gender. Hence, new alternative formulas for CL_{cr} were deduced by incorporating one or more of these factors as variables, along with SCr (in mg/100 mL), but didn't require the collection of urine specimens. Although UCr is not included in the final versions of the alternative formulas, a common step in deducing the formulas required the determination of the 24-hour urinary creatinine concentrations. A comprehensive explanation of all the steps involved in the deduction of these formulas is not included here as it is beyond the scope of this present

work. However, a brief description of some widely accepted formulas for CL_{cr} and their variables is presented below.

In 1973, Roger W. Jelliffe [51] deduced two formulas for determining CL_{cr}: first, a non-normalized gender-based formula for CL_{cr} (in mL/min) by incorporating age, and second, a normalized gender-based formula for CL_{cr} (in mL/min/1.73 m^2) by incorporating both age and BSA (expressed in m^2), as represented by Equation 1.4 and Equation 1.5 respectively.

$$\mathrm{CL_{cr}\ (for\ males)} = \frac{98 - [0.8 \times (\mathrm{Age} - 20)])}{\mathrm{SCr}}$$

(Eq. 1.4)

$$\mathrm{CL_{cr}\ (for\ males)} = \frac{\{98 - [0.8 \times (\mathrm{Age} - 20)\} \times \left(\frac{\mathrm{BSA}}{1.73}\right)}{\mathrm{SCr}}$$

(Eq. 1.5)

CL_{cr} for females can be determined using Jelliffe's equation by multiplying CL_{cr} obtained from Equation 1.4 and Equation 1.5 with 0.90. The age of the individual whose CL_{cr} has to be determined must be rounded off to the nearest digit divisible by 10 in these equations.

In 1976, Donald W. Cockcroft and M. Henry Gault [52] incorporated BW (in kg) and age to deduce a gender-based formula to determine CL_{cr} (in mL/min), as represented by Equation 1.6.

$$\mathrm{CL_{cr}\ (for\ males)} = \frac{(140 - \mathrm{Age}) \times (\mathrm{BW})}{72 \times \mathrm{SCr}}$$

(Eq. 1.6)

CL_{cr} for females can be determined using the Cockcroft-Gualt equation by multiplying CL_{cr} obtained from Equation 1.6 with 0.85. Amongst the CL_{cr} determining formulas, the Cockcroft-Gault equation has been extensively applied in the literature [53-57].

In 1988, Daniel E. Salazar and George B. Corcoran [58] incorporated BW (in kg), Ht (in m) and age to deduce gender-based formulas to determine CL_{cr} (in mL/min), specifically for obese individuals, as represented by Equation 1.7 and Equation 1.8.

$$\mathrm{CL_{cr}\ (for\ males)} = \frac{(137 - \mathrm{Age}) \times [(0.285 \times \mathrm{BW}) + (12.1 \times \mathrm{Ht^2})]}{(51 \times \mathrm{SCr})}$$

(Eq. 1.7)

$$\mathrm{CL_{cr}\ (for\ females)} = \frac{(146 - \mathrm{Age}) \times [(0.287 \times \mathrm{BW}) + (9.74 \times \mathrm{Ht^2})]}{(60 \times \mathrm{SCr})}$$

(Eq. 1.8)

All the new CL_{cr} determining formulas mentioned above have a common limitation, as these formulas apply to individuals with stable levels of SCr [48]. Hence, in 2002, Roger Jelliffe [48] reported another formula to calculate CL_{cr} (in hundreds of mL/min) for individuals with unstable levels of SCr, by incorporating BW (in hundreds of grams), adjusted creatinine production rate (P_{adj}, expressed in mg/day) and recording two serum creatinine concentrations (SCr_1 and SCr_2, expressed in mg/100 mL) after a period of T (expressed in number of days), as represented by Equation 1.9.

$$\mathrm{CL_{cr}} = \frac{(\mathrm{P_{adj}} \times \mathrm{T}) - 0.4\mathrm{BW}(\mathrm{SCr_2} - \mathrm{SCr_1})}{\mathrm{T} \times \mathrm{SCr_{avg}} \times 1440}$$

(Eq. 1.9)

In Equation 1.9, SCr_{avg} is the average of SCr_1 and SCr_2, and 1400 is the minutes in 24 hours. In this 2002 Jelliffe formula, P_{adj} was calculated by equating it with 24-hour urinary creatinine concentration [48].

On the other hand, based on the determination methods, GFR can be broadly classified as the measured GFR (mGFR) and the estimated GFR (eGFR). While mGFR can be determined by assessing the clearance of exogenous filtration markers like inulin, iohexol, chromium 51-ethylenediamine tetraacetic acid, technetium 99m diethylenetriamine pentaacetic acid and iothalamate, eGFR can be determined by assessing the clearance of endogenous filtration markers like serum creatinine and cystatin C [59]. Due to the impractical and expensive approach to determining mGFR, the determination of eGFR, despite its lack of precision, has found prominence in clinical practices [59]. At this point, a similarity between the calculated eGFR (based on the clearance of creatinine) and CL_{cr} can be reckoned, and the terms are often used interchangeably. For example, there are reports where the Cockcroft-Gault equation is stated to be used to determine GFR [60, 61]. However, it is important to note that although CL_{cr} provides a close evaluation of eGFR, the values obtained for simultaneously recorded

CL_{cr} and eGFR are unlikely to be equal for an individual with stable SCr. A study in 2011 reported the overestimation of GFR by using the Cockcroft-Gault equation due to the tubular excretion of creatinine [62]. Another analysis, published in the year 2006, reported the underestimation of GFR by 14 % in normal-weight diabetic patients, and overestimation of GFR by 15 % and 55 % in overweight and obese diabetic patients respectively, by using the Cockcroft-Gault equation [63]. In 1958, Edwards and Whyte reasoned out that the value obtained for CL_{cr} is 'equivalent' to that of GFR [49]. In 2016, *Fernandez-Prado et al.* also rightly distinguished between CL_{cr} and eGFR, as they compared the Cockcroft-Gualt equation with the equation for eGFR proposed by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [64].

$$\text{eGFR} = 142 \times \min\left(\frac{\text{SCr}}{\kappa}, 1\right)^{a_1} \times \max\left(\frac{\text{SCr}}{\kappa}, 1\right)^{a_2} \times 0.9938^{\text{Age}} \times 1.012 \text{ (if female)}$$

(Eq. 1.10)

Equation 1.10 represents the equation for eGFR (in mL/min/1.73 m^2) proposed by CKD-EPI in 2021 where SCr is expressed in mg/100 ml; κ is 0.9 for males and 0.7 for females; min(SCr/κ, 1) refers to the minimum of SCr/κ and 1; max(SCr/κ, 1) refers to the maximum of SCr/κ and 1; the coefficient a_1 (-0.302 for males and -0.241 for females) is used when SCr for males is ≤ 0.9 mg/100 mL for males and ≤ 0.7 mg/100 mL for females; and the coefficient a_2 (-1.200) is used when SCr is > 0.9 mg/100 mL for males and > 0.7 mg/100 mL for females [65]. This 2021 CKD-EPI equation is an updated version of another equation proposed by CKD-EPI in 2009, which was race-dependent. The task force formed by the National Kidney Foundation (NKF) and the American Society of Nephrology (ASN) recommends the 2021 CKD-EPI equation over the 2009 CKD-EPI equation, ruling out the race factor from the equation [66].

Apart from CKD-EPI equation, some formulas proposed by the Modification of Diet in Renal Disease (MRDR) study for calculating eGFR (in mL/min/1.73 m^2) are also popularly used. The formulas for eGFR proposed by the MRDR study are based on age, gender, demography and several serum and urine variables (SCr, concentration of serum urea nitrogen and albumin and urine urea nitrogen excretion) [67-69]. Later, a simplified MRDR equation, named the abbreviated MRDR (aMRDR) equation, was deduced [69]. Equation 1.11 represents the aMRDR equation, which depends on SCr (in mg/100 mL),

age, gender and ethnicity. The MDRD equations, however, faced criticisms due to poor precision and performance in calculating eGFR > 60 mL/min/1.73 m^2 [70].

$$\text{eGFR} = 186 \times \text{SCr}^{-1.1554} \times \text{Age}^{-0.203} \times 0.742 \text{ (if female)} \times 1.21 \text{ (if African} - \text{American)}$$

(Eq. 1.11)

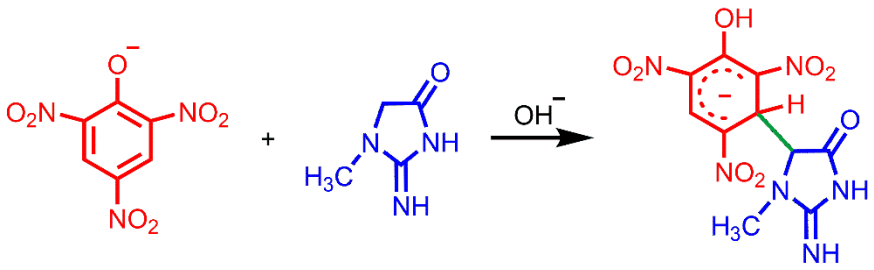
With eGFR unit expressed in mL/min/1.73 m2, according to the published guidelines by Kidney Disease: Improving Global Outcomes (KDIGO), eGFR ≥ 90 (G1 stage) is considered normal or high; 60 ≤ eGFR ≤ 89 (G2 stage) refers to a slight decline in renal function; 45 ≤ eGFR ≤ 59 (G3a stage) refers to a mild or moderate decline in renal function; 45 ≤ eGFR ≤ 59 (G3b stage) refers to a moderate or severe decrease in renal function; 15 ≤ eGFR ≤ 29 (G4 stage) refers to a critical decline in renal function; and, eGFR ≤ 15 (G5 stage) refers to renal failure [71, 72]. While it can be inferred that any individual who is at the G3a, G3b or G4 stage, is suffering from kidney disease, the G2 stage infers the early stage of renal issues, and patients at the G5 stage require dialysis. Notably, although the lower eGFR has conventionally been associated with renal malfunctions, high eGFR might also indicate hyperfiltration of blood due to any possible renal injury [73].

1.3 Creatinine determination methods used in clinical practices and their limitations

As the previous sections highlighted the importance of creatinine, it is comprehensible now that the accurate determination of creatinine concentration in human body fluids is necessary to well-utilize its role in monitoring human health (renal function). Apart from accuracy, the other aspects that must be considered in developing creatinine quantification methods are sensitivity, robustness, longevity, cost and time. Since the late 19th century, several methods have been proposed for creatinine detection and quantification in urine and serum. However, only two methods, the 'Jaffe method' and the 'Enzymatic method', are popularly used in clinical practices. A brief description of the development and principles of both methods, along with a comparison of their analytical performances, is provided in the sub-sections below.

1.3.1 Jaffe method

The Jaffe method is regarded as the traditional method for determining creatinine levels. It is based on the reaction between creatinine and sodium picrate in an alkaline medium to form an orange-red coloured creatinine-picric acid complex (Janovsky complex). It was in 1886 when Max Jaffe studied the behaviour of picric acid in human urine and reported that creatinine reacts with picric acid in the presence of a few drops of dilute potassium and sodium hydroxide solution to form the coloured complex [74]. Although Jaffe established the principle, Otto Folin and Morris developed the reaction for clinical practices by mixing 1 mg of creatinine with 20 mL of picric acid and 1.5 mL of 10 % sodium hydrate solution.[75]. The mixture was incubated for 10 minutes before subjecting it to a colorimeter [75, 76]. As the colour of the complex was noted to be more intense for samples with higher concentration of creatinine, the complex exhibited an absorption peak at 520 nm with its intensity proportional to creatinine concentration [74, 77, 78]. Thus, a colorimetric determination of creatinine could be carried out using the Jaffe reaction. Scheme 1.2 is a structural representation of the Jaffe reaction. The structure of the Janovsky complex, as shown in Scheme 1.2, is well-established in the literature [79, 80].



Picrate ion **Creatinine** **Janovsky complex**

Scheme 1.2: Structural representation of the Jaffe reaction.

The wide acceptance of the Jaffe reaction in clinical practices had ceased the clinical use of the Neubauer reaction for creatinine determination, which was proposed in the late 19th century and was based on a creatinine-zinc coloured complex formation in an alcoholic medium [81]. However, the major drawback of the Jaffe reaction for creatinine determination was the non-specificity of the reaction, as Max Jaffe himself noted that the formation of the Janovsky complex was affected in the presence of organic compounds

like glucose and acetone [74, 81]. Further studies confirmed that other body fluid components like protein, glucose, bilirubin, uric acid, antibiotics, etc. or high doses of administered aminoglycoside antibiotics like streptomycin interfere with the Jaffe reaction [82-85].

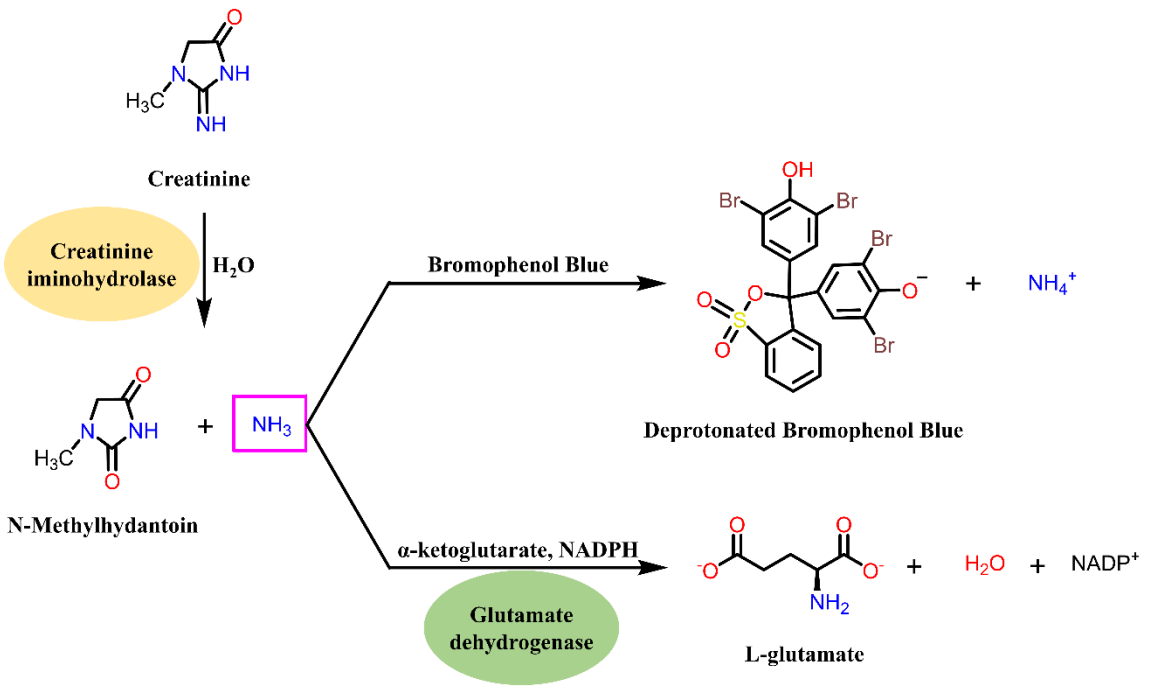
Several modifications of the Jaffe reaction have also been proposed to minimize the effect of interference. The kinetic study of the Jaffe reaction proposed by J. G. H. Cook emerged as a notable modification of the traditional method [86]. Cook recorded the increase in the absorption peak at 510 nm for the system containing standard creatinine solution/serum and Jaffe reagents, at a time interval of 60 s, and the kinetic study revealed that the rate of increase of the absorption peak is proportional to the creatinine concentration [86]. Another interesting modification of the Jaffe reaction involves an additional step of deproteinizing the serum before adding the Jaffe reagent, as the use of sodium dodecyl sulphate, tungstic acid and potassium ferricyanide has been reported for the removal of proteins from the sample [87-91]. In 1935, Borsook also demonstrated the use of Lloyd's reagent to adsorb creatinine from acid serum or plasma, followed by its elution in an alkaline medium and determination via the Jaffe reaction [92]. These modified methods were not free from limitations either. While the Kinetic Jaffe method suffers from imprecision at low creatinine levels, the deproteinization step doesn't assure the minimisation or removal of interference by other body fluid components, and the process of adsorption by Lloyd's reagent, although ensures higher specificity, results in the loss of creatinine in the subsequent elution step [93, 94]. An electrochemical study of the Jaffe reaction with Edge Plane Pyrolytic Graphite electrode and screen-printed carbon electrode was also reported with its application restricted to detecting only urinary creatinine [79].

Nevertheless, despite some limitations, the Jaffe method is the oldest method that is still used in clinical practices for determining creatinine levels.

1.3.2 Enzymatic method

The use of enzymes for determining creatinine levels stemmed from the attempt to develop interference-free methods as an alternative to the Jaffe method, and it succeeded to a great length. It was especially in the early 1980s when several promising enzymatic methods, using spectroscopic techniques, were reported. Initially, the enzyme 'creatinine

iminohydrolase' was preferred, which hydrolyses creatinine to produce N-methylhydantoin (IUPAC: 1-methylimidazolidine-2,4-dione) and ammonia [95-97]. *Sunberg et al.* used creatinine iminohydrolase to develop an enzymatic creatinine determination method by mixing the produced ammonia with bromophenol blue indicator and analysing the change in the absorption band at 600 nm, which was proportional to creatinine concentration [95]. *Toffaletti et al.* also utilized the same reaction scheme and studied the difference in reflection density at 600 nm at an angle of 45°, which was dependent on creatinine concentration [96]. *Tanganelli et al*., although used creatinine iminohydrolase to yield N-methylhydantoin and ammonia, the produced ammonia was subjected to an auxiliary reaction with α-ketoglutarate, catalysed by glutamate dehydrogenase, in the presence of NADPH [97]. The auxiliary reaction produces L-glutamate and $NADP^+$, with the concentration of $NADP^+$ proportionally varying with creatinine concentration in the sample [97]. After an incubation period of 15 min, the change in the absorbance at 340 nm was recorded to determine creatinine concentration [97]. Scheme 1.3 illustrates the reported reaction steps for the enzymatic creatinine determination using the creatinine iminohydrolase enzyme [95-97].

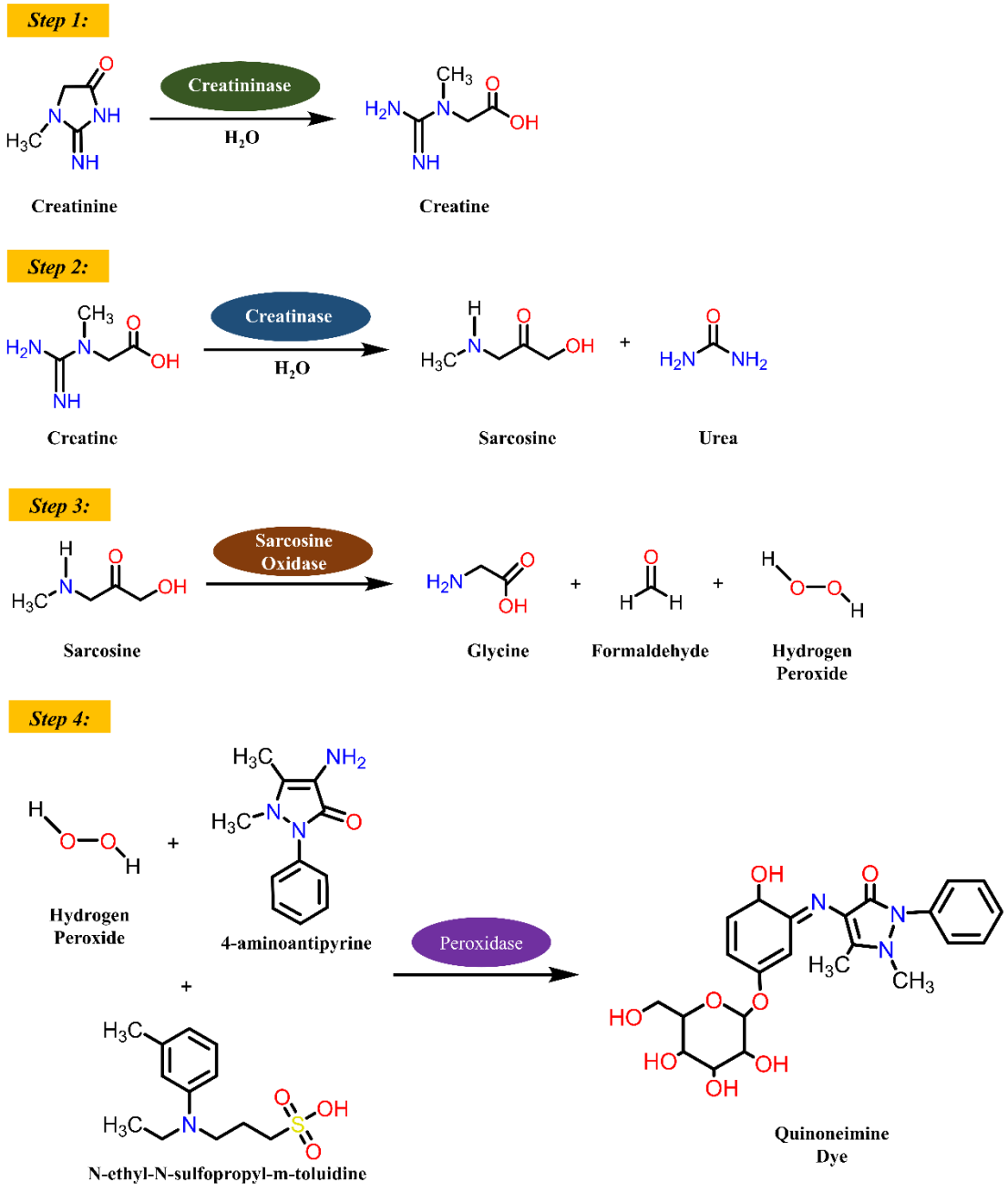


Scheme 1.3: Enzymatic hydrolysis of creatinine by 'creatinine iminohydrolase' and the subsequent steps for creatinine determination.

It can be seen that the enzymatic determination of creatinine using creatinine iminohydrolase is dependent on the produced ammonia. Thus, interference by endogenous ammonia is a major drawback of this system. Although *Tanganelli et al*. mentioned the necessity of a preincubation stage for eliminating endogenous ammonia, where the auxiliary reaction is carried out without adding the enzyme to the system, this is a cumbersome and time-consuming process that cannot be apt for clinical practices [97]. Hence, researchers deflected their attention to developing enzymatic creatinine-determining methods that can be labour-saving and independent of any endogenously present component.

The exploration of the enzyme, creatininase (creatinine amidohydrolase), resolved the complication mentioned above to a large extent. Concomitantly, a few enzymatic creatinine-determining methods were reported, based on the initial hydrolysis of creatinine by creatininase to produce creatine, followed by different subsequent enzyme-catalysed steps [78, 98, 99]. However, in all these methods, the role of ammonia in determining creatinine level is completely obliterated and no additional preincubation step is required. Such advantageous observations eventually led to the development of an automated and sensitive multi-enzyme, multi-step process for creatinine determination, that was also accepted for clinical practices. The reactions occurring in this clinically practiced enzymatic method can be explained in 4 steps: ‘Step 1’ is the hydrolysis of creatinine by creatininase to produce creatine; ‘Step 2’ is the hydrolysis of creatine by creatinase to produce sarcosine and urea; ‘Step 3’ is the oxidation of sarcosine by sarcosine oxidase to produce glycine, formaldehyde and hydrogen peroxide; and ‘Step 4’ is the reaction of hydrogen peroxide with 4-aminoantipyrine and N-ethyl-N-sulfopropyl-m-toluidine, catalysed by peroxidase, to produce an quinoneimine dye [78]. Thus, a colorimetric creatinine determination can be accomplished as the formation of the dye results in a change of absorbance at 548 nm, which is proportional to creatinine concentration [78]. Interestingly, *Fossati et al.* presented a modified version of ‘Step 4’ where 3,5-dichloro-2-hydroxybenzenesulfonic acid is used in place of N-ethyl-N-sulfopropyl-m-toluidine to yield a quinine-monoimine dye [99]. The change in absorbance at 510 nm was studied in the Fossati method and the absorbance change was noted to be proportional to creatinine concentration [99]. However, HCl was also produced as a by-product in ‘Step 4’ of the Fossati method, which might also interfere with the system by decreasing the activity of

enzymes. The reaction steps involved in the clinical method of enzymatic determination of creatinine are illustrated in Scheme 1.4 [78].



Scheme 1.4: Enzymatic hydrolysis of creatinine by 'creatininase' and the subsequent steps used in clinical practises for creatinine determination.

Many reports have asserted that the enzymatic method is interference-free and must be preferred over the Jaffe method. However, some recent studies have revealed

contradictions. Although to a lesser extent, as compared to Jaffe, it has been confirmed that protein and glucose interfere with the results of the enzymatic method [100]. Interference by components like lithium heparin (a common additive in blood collection tubes) was also reported [100]. Another general challenge associated with the enzymatic processes is maintaining the stability and activity of the enzymes.

1.3.3 Jaffe v/s Enzymatic: A comparison of the methods

The choice between applying the Jaffe method or the Enzymatic method is at the discretion of the particular laboratory. While some laboratories prefer the Enzymatic method for its higher sensitivity and selectivity, some opt for the Jaffe method due to its simplicity and cost-effectiveness, compared to the complicated and expensive enzymatic method.

Küme et al. [78] applied both clinically accepted methods, by using Architect c16000 auto-mated analyzer (Abbott Diagnostics Inc, Park City, IL, USA), in a large sample of urine and serum specimens. 0.6, 1.6 and 6.0 mg/dL for serum creatinine, and 30, 60 and 120 mg/dL for urinary creatinine were explored in the study [78]. The analytical performances of the methods as determined by *Küme et al.* [78] are tabulated in Table 1.1. It can be known from the comparative analysis of the analytical performances that the limit of detection (LOD) and limit of quantification (LOQ) obtained by the Jaffe and the Enzymatic method were determined to be equal in serum. However, in urine, the LOD and LOQ obtained by the Enzymatic method, were lower than the corresponding values obtained by the Jaffe method, implying the Enzymatic method to be more sensitive at lower concentrations.

Table 1.1: Comparison of the analytical performances of the Jaffe method and the Enzymatic method in serum and urine.

	Jaffe method		**Enzymatic method**	
Kit used	Creatinine (Jaffe), catalog no. 7D64- 20		Multigent Creatinine (Enzymatic), catalog no. 8L24- 31	
Real sample	Urine	Serum	Urine	Serum
LOD in mg/dL	0.25	0.01	0.07	0.01

***LOQ in mg/dL**	2	0.05	0.5	0.05
Detection Range in mg/dL	0.21-37.85	0.20-38.70	0.10-40	0.25-40
** LOQ was calculated as the minimum concentration at which the coefficient of variations is lower than 10 %*				

On comparing the samples having low creatinine concentrations, it was found that the Jaffe method calculates 7 % higher values compared to the Enzymatic method [78]. However, in another comparative analysis of the methods applied to the serum samples of 529 patients, as presented by *Schmidt et al*. [101], it was found that the Jaffe method has higher precision and lower coefficient of variations than the Enzymatic method. So, there are statistical differences between both methods but there is no other gold standard to compare and claim higher accuracy for one method over the other.

Thus, it can be stated that although the Jaffe method and the Enzymatic method have found prevalence in clinical practices, the methods have limitations and inadequacies. Hence, the route stays unlatched for researchers to explore newer ideas and approaches to either alter the clinically practised methods for betterment or to develop alternative, sensitive, selective and robust creatinine determination methods.

1.4 Coordination with transition metal ions: the intrinsic property of creatinine

The compound, creatinine, can act as a ligand with multiple coordinating sites (the oxygen atom of the carbonyl group, the endocyclic nitrogen atom at position 1 and the exocyclic nitrogen bonded to the carbon atom at position 2) and form complexes with transition metal ions. It was during the latter half of the 20th century when the complexation of creatinine with transition metal ions was extensively explored [102-110]. Several complexes of creatinine with copper [102, 103], cobalt [104], platinum [105, 106], palladium [105, 106], nickel [107], zinc [108], cadmium [108], and mercury [108, 109] were reported. The nature of these complexes was studied in different mediums such as water, ethanol-water, methanol, methanol-water, alcohol-water-HCL, perchloric acid, pyrrole (Py), DMSO, etc. Interestingly, it was established that the ratio of creatinine-metal in the complexes, the presence of other ligands in the coordinating sphere, and the

coordinating sites of creatinine depend largely on the solvent system, reflux time and temperature (if required), and the pH [110]. As researchers continue to design new methods to detect creatinine and determine its level in body fluids, it is noticed that this intrinsic property of the compound has often been utilized to play the most significant role and lay the foundation of the methods. Thus, due to the importance of creatinine-transition metal complexes in creatinine sensor development, a keen interest among researchers is still seen, to tune the conditions and synthesise new complexes [111-113].

In the complexes, while the coordination of creatinine via its endocyclic nitrogen atom (*ortho* to the carbonyl group) is most commonly observed, there are some reported complexes where along with the endocyclic nitrogen atom, the exocyclic nitrogen atom or carbonyl group also participate in coordination. The coordinating sites of creatinine and creatinine-metal ratio in some of the reported complexes of creatinine with different transition metal ions, and the respective solvent system, are summarised in Table 1.2.

Table 1.2: Different coordinating sites and creatinine-metal ratios reported for creatinine-transition metal complexes in various solvent systems.

Creatinine complex with-	**Solvent**	**Creatinine-metal ratio in the obtained complex**	**Coordinating sites of creatinine**	**Reference**
Copper	Water	2:1	Endocyclic nitrogen and carbonyl group	[102]
Copper	Methanol	4:1	Endocyclic nitrogen	[103]
	Acetonitrile	3:1 (Refluxed at 50-60 °C under constant stirring for 1 hour)		

		2:1 (Refluxed at 50-60 °C under constant stirring for 6-7 hours)		
Cobalt	Water-methanol	2:1	Endocyclic nitrogen	[104]
Platinum	Water	1:2	Endocyclic nitrogen and carbonyl group	[105]
	Water (in argon environment)	3:1		
Palladium	Water-NaOH	2:1		
Zinc	Water-methanol	2:1	Endocyclic nitrogen	[108]
Cadmium	Water-methanol	2:1 (with chloride and bromide salts of cadmium)		
		1:1 (with iodide salt of cadmium)		
Mercury	Water-methanol	2:1		
Mercury	Water-ethanol-HNO_3	1:2	Endocyclic nitrogen and exocyclic nitrogen	[109]

It is also important to note that there are several proposed tautomeric forms of creatinine (I-IV), as shown in Figure 1.1. However, it has been observed that most of the creatinine-transition metal ion complexes have been reported with the tautomeric forms, I and III, of creatinine.

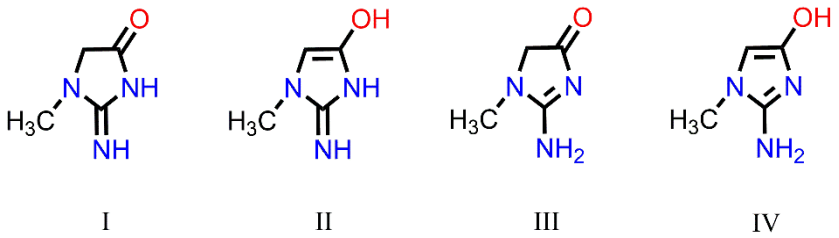


Figure 1.1: Tautomeric forms of creatinine.

1.5 Development of new creatinine sensors

Several new creatine sensors have been reported, especially in the last couple of decades, that have enriched the scientific community with exciting findings. Another noteworthy aspect regarding the new creatinine sensors is the use of different analytical methods as transduction systems. Earlier, only the colorimetric methods were predominantly employed to design creatinine determination methods, as was the case of the Jaffe method and the Enzymatic method used in the clinical practices too. Eventually, other transduction systems, such as fluorescence [114-122], Raman spectroscopy [123-126], electrophoresis [127-129], chromatography tandem-mass spectroscopy [130-133], and electrochemistry [134-157], were also utilized, apart from some new colorimetric methods [158-163] being proposed. Amongst these, electrochemical sensors are claimed to have several advantages such as lower cost, higher sensitivity and selectivity, portability, ease of operation, time-saving and robustness [164]. Although it can be argued that the other transduction system might also have several benefits to offer, what gives an edge to electrochemical sensors, as rightly pointed out by *Baranwal et al.* [165], is the variability of the output signals in the form of voltage, current, electrochemical impedance, etc. and its low theoretical detection limits.

In a broader sense, based on the mode of action, all creatinine determination methods can be classified into two categories: a) Enzymatic process, and b) Non-enzymatic process. These two terms are readily comprehensible. Although the enzymatic processes accelerate the sensing reactions and are likely to offer higher selectivity, enzymes are expensive and sensitive to varying physiological conditions (pH and temperature) [166]. On the other hand, the non-enzymatic processes are comparatively

more challenging to design for selective and precise sensing but usually offer long-term stability and are mostly cheaper than enzymatic processes.

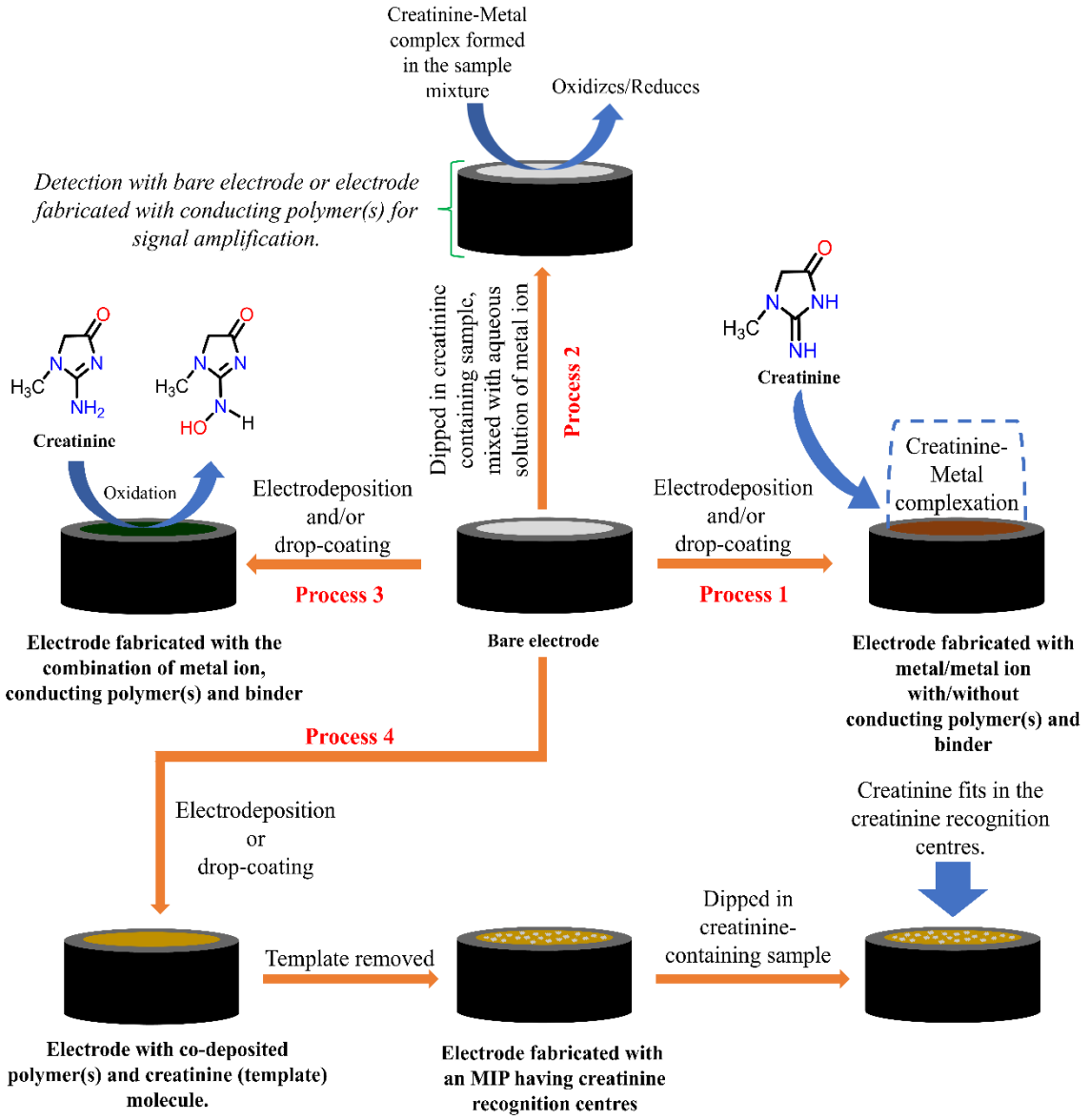


Figure 1.2: Illustration of the detection principles of different methods for non-enzymatic electrochemical determination of creatinine.

As this thesis focuses on developing novel non-enzymatic electrochemical creatinine determination methods, understanding the working principles and detection techniques of some of the recently reported non-enzymatic electrochemical creatinine sensors is required. Prior to that, it is important to note a property of creatinine from the electrochemical point of view. It has been widely reported that creatinine is an

electrochemically inactive molecule [155, 156], that is, it doesn't exhibit any peak at any potential on the bare electrode surface. Hence, most electrochemical creatinine detection methods are based on transforming creatinine into electroactive components by coordination with transition metal ions. When the electrode surfaces are deposited with metal ions or metal, it offers a platform for the creatinine-transition metal ion complexation. Due to this complexation, a shift in the potential and (or) change in the amplitude of the native redox peaks of the metal ions are (is) usually observed, as has been determined by *Raveendran et al*. [143] who analyzed the change in voltammogram responses of creatinine solution with a copper electrodeposited screen-printed electrode. It is found that such differences in the electrochemical response vary quantitatively with creatinine concentration and form the base for determining creatine levels in body fluids. The detection principle of such methods is illustrated in 'Process 1' of Figure 1.2.

Copper is the most common transition metal with which, the complexation of creatinine has been explored for sensor development by several researchers [136-143]. *Jankhunthod et al.* reported a creatinine determination method, validated in synthetic urine, by carrying out electrodeposition of copper on the surface of graphite screen-printed electrode and establishing the difference between the cyclic voltammogram responses of buffer solutions in the absence and presence of creatinine respectively [136]. The physical stability of the deposited materials is often enhanced with binders like gelatin, Nafion, etc. *Sato et al.* reported a copper electrodeposited gold electrode, drop-coated with Nafion solution as the binder, for the electrochemical determination of creatinine [140]. Meanwhile, it has also been established that electrode materials in nano-dimensions improve the performance of the creatinine sensors [157]. Based on the same creatinine-transition metal ion principle, *Kumar et al.* [138] demonstrated a highly sensitive and selective creatinine determination method in human serum, using a screen-printed carbon electrode, modified with cuprous oxide nanoparticles functionalized to a zwitterion. Furthermore, polymers are often introduced as supporting matrices for the metal ions on the sensing platform. Conductive and electroactive carbon-based materials like reduced graphene oxide (rGO), polypyrrole (PPy), polyaniline (PANi), carbon black (CB), carbon nanotubes (CNTs) etc. or their combinations are also used for amplification of the redox peaks, besides being used as support. For example, *Gao et al.* [142] used the combination of copper nanoparticles with rGO and polydopamine for electrode modification to design

a creatinine sensor. Apart from copper, iron ions in combination with different materials, such as Fe^{3+}/CB [144] and Fe^{3+}/cotton fibre membrane [145] have been another choice as electrode materials for the researchers to design creatinine sensors. Researchers also suggested a modification in this technique by utilizing the synergistic effect of combining two metals (metal ions), such as Ag/Fe [146], Au/Ag [147], and Cu_2O/Au [148]. The synergistic effect of using two metals is produced in different ways. Mahmoud et al. observed two distinct anodic peaks for Ag^+/Ag and Fe^{3+}/Fe^{2+} redox processes respectively [146]. With the increase in creatinine concentration, while the intensity of the anodic peak for Ag^+/Ag (I_{Ag}) decreased, the intensity of the anodic peak for Fe^{3+}/Fe^{2+} (I_{Fe}) remains almost same, thus, indicating a stronger coordination between creatinine and Ag^+. Mahmoud et al. reported better performance of the designed sensor, by enabling a ratiometric detection (I_{Ag}/I_{Fe}) of creatinine [146]. *Nene et al.* [147] reported a creatinine sensor, based on the chemisorption of creatinine via its N groups to Au(0) and Ag(0) which decreases the intensity of a conditionally produced single oxidation peak observed for both metals.

Notably, electrode modification with metal ions is not necessary to design a creatinine sensor based on creatinine-transition metal ion complexation. *Kaewket and Ngamchuea* [137] reported an interesting process to determine creatinine level in synthetic urine by adding $CuSO_4$ solution to the sample directly, thus, allowing the creatinine-copper complex in the sample itself, and recording the change in the voltammogram responses. Measures for prior removal of anions from the synthetic urine sample have to be taken in this process, as the metal cation could also form precipitates with phosphate and carbonate anions present in the sample [137]. The detection principle of such methods is illustrated in 'Process 2' of Figure 1.2.

However, although very rare, the direct oxidation of creatinine on modified electrode surfaces has been reported in the literature, which somewhat contradicts the popularly accepted electrochemical inactivity of the molecule [134, 135]. While *Fekry et al.* [135] reported the combination of CNT, folic acid and Ag-nanoparticles, *Singh et al.* [135] reported the combination of Fe, Cu and rGO to modify the surface of the respective electrodes. The mechanism of direct electrochemical oxidation suggests the oxidation of the exocyclic amino group of creatinine to hydroxylamine, as the tautomeric form III of creatinine (shown in Figure 1.1) was considered to explain the mechanism. A certain

ambiguity does arise, whether the change in the voltammogram responses is truly due to the electrochemical oxidation of the molecule or if the metal ions on the electrode surface have any role to play. The detection principle of such methods is illustrated in 'Process 3' of Figure 1.2.

While the methods based on the creatinine-transition metal ion complexation have been leading in the non-enzymatic electrochemical creatinine determination process, another method opted by some researchers which has gained popularity too, is molecular imprinted polymer (MIP) based. The steps involved in the MIP-based creatinine determination processes can be divided into 3 general steps: a) co-deposition of a polymer and the template molecule (creatinine) on the electrode surface, b) fabricating the MIP by removing the template molecule from the electrode surface which creates creatinine-shaped holes on the polymer, and c) recording the voltammogram or impedance of creatinine containing samples with the MIP-fabricated working electrode. The detection principle is based on the ability of the creatinine-shaped cavities on the polymer matrix to act as creatinine recognition centres. So, when the MIP-fabricated working electrode is dipped into samples containing creatinine, the molecule fits in the recognition centres, like the 'lock and key' model of enzyme action. This causes a change in the voltammogram or impedimetric response, which is proportional to the creatinine concentration. The detection principle of such methods is illustrated in 'Process 4' of Figure 1.2.

The co-deposition of the polymer and the template molecule on the electrode surface can be accomplished in two ways: i) direct electro-polymerization of the monomer by taking a solution of the monomer mixed with the template molecule, and ii) drop-coating a polymeric solution on the electrode surface, after preparing the polymer in an ex-situ process in the presence of the template molecule. Both ways can lead to the fabrication of the electrode surface with the template-trapped polymer layer. The bonding between the monomeric group and the template molecule in the co-deposited polymer-template layer can be either covalent or non-covalent (via hydrogen bonding, dipole-dipole interaction, van der Waals force, etc.) [167]. *Sajini and Mathew* [167] pointed out that while covalent bonding can result in more specific and well-defined recognition centres after template removal, non-covalent bonding requires simpler synthetic procedures and the template molecule can be extracted easily. *Li et al.* [149] reported electrode fabrication with a creatinine-imprinted polydopamine layer, in combination with graphene

nanoplatelets, for the electrochemical determination of creatinine. Stirring in water and HCl was enough to overcome weak intermolecular interactions between creatinine and the monomeric groups and remove creatinine to form the MIP in the reported work [149]. On the other hand, *Prabhu et al.* [150] reported a reflux treatment in acetic acid to break the comparatively stronger H-bonding between creatinine and the monomeric unit of methacrylic acid in their reported MIP-based creatinine determination method.

Although the discussion above primarily focussed on non-enzymatic electrochemical creatinine sensors, It can be understood that designing new creatinine determination methods which can overcome the limitations of the clinically practised methods is a prime requisite and indeed a field of great interest for researchers in different corners of the world. In fact, creatinine is the most explored biological component after glucose in the sensor development area. The underlying mammoth challenge, however, can easily be sensed as both Jaffe and Enzymatic methods were developed into practical use many decades ago in the 20th century and, despite their limitations, no other proposed method could find prominence and wide acceptance yet. For a method to topple the long-accepted clinically practised methods, it must be of superior quality on all fronts-precision, selectivity, LOD, cost, time, etc. Nevertheless, the endeavour continues with a grander perspective now. Today, the task for the researchers is not merely to overcome the previous limitations but to develop their creatine sensor into a point-of-care-testing (POCT) device. A POCT device for determining creatinine level can help patients needing frequent monitoring of their creatinine level to accurately access their renal function without visiting clinics every time.

1.6 Importance of creatinine sensors in the future: a forecast

Some recent studies reported that 700 million people in the world suffer from CKD and with the inclusion of acute kidney injury (AKI) and renal failure cases, the number stands at a staggering 850 million which is more than 10% of the world population [168, 169]. While, the most common causes of CKD include hypertension, diabetes, genetics, obesity, malnutrition, use of tobacco products, exposure to drastic environmental changes, etc., a study on the role of lifestyle and economic perspective revealed that CKD has a much larger prevalence in low-income countries (LICs) and lower-middle-income countries (LMICs) [169]. Globally, there has been a 33% increase in CKD cases between

1990-2017, with almost one-third of the patients from India and China alone [169]. It has also been projected that by 2040, CKD will globally be the 5th leading cause of years of life lost (YLLs) after ischaemic heart diseases (IHD), stroke, lower respiratory infections (LRIs) and chronic obstructive pulmonary disease (COPD) [170].

If the importance of accurate creatinine sensors in the future has to be forecasted, the numbers on which the prediction can be based would be the rising cases of kidney diseases. With creatinine promising to stay the most reliable renal function marker, the dependence on creatinine sensors is only likely to increase in the time ahead.

1.7 Aim and objectives of the work

Aim: To explore new approaches and develop novel non-enzymatic electrochemical methods for accurately determining creatinine levels in human serum and urine.

To meet the aim of this work, the following objectives are fulfilled:

- ✓ A urinary creatinine determination protocol is developed by chemical transformation of creatinine to an electro-active species in a metal-free condition.
- ✓ Coordination of cobalt ion with creatinine and 2-nitrobenzadehyde (2-NBA) is established.
- ✓ A serum creatinine determination method is developed based on the complexation property of creatinine with 2-NBA coordinated cobalt ion.
- ✓ A new MIP platform is fabricated for creatinine determination, verified by the creatinine-copper interaction.
- ✓ Interference studies are carried out for the new creatinine determination methods.
- ✓ The methods are validated in real media.

1.8 Plan of work

- ❖ To study the electrochemical response of creatinine and 2-NBA mixture in buffer, in the presence of an alkali (NaOH), with bare glassy carbon electrode (GCE).
- ❖ To establish the formation of electroactive species in the creatinine-2-NBA-NaOH system.
- ❖ To identify the creatinine concentration-dependent redox peak in the creatinine-2-NBA-NaOH system.

- ❖ To optimize the conditions (pH, reaction time and concentration) of the creatinine-2-NBA-NaOH system for efficiently determining creatinine levels.
- ❖ To study the creatinine-2-NBA-NaOH system in the presence of other urinary components (glucose, uric acid, ascorbic acid, dopamine and urea).
- ❖ To demonstrate the creatinine-2-NBA-NaOH system for accurate electrochemical determination of urinary creatinine.
- ❖ To propose a plausible mechanistic pathway of the reaction between creatinine and 2-NBA in the alkaline medium and designate all the redox peaks, supported by electrochemical and spectroscopic findings.
- ❖ To analyse the alteration in the creatinine-2-NBA reaction by adding cobalt ions to the system.
- ❖ To optimize the conditions to yield creatinine-cobalt and creatinine-cobalt-2-NBA coordination complexes
- ❖ To characterize the coordination complexes with microscopic, spectroscopic and electrochemical techniques.
- ❖ To distinguish the physiochemical differences in the coordination complexes.
- ❖ To study the electrochemical response of creatinine, cobalt ion and 2-NBA mixture in buffer with bare GCE.
- ❖ To identify the change in voltammogram responses, proportional to the creatinine concentration, due to coordination in the creatinine-cobalt-2-NBA system.
- ❖ To optimize the conditions (pH, reaction time and concentration) of the creatinine-cobalt-2-NBA system for efficiently determining creatinine levels.
- ❖ To study the creatinine-cobalt-2-NBA system in the presence of other serum components (uric acid, ascorbic acid, glucose, urea and albumin).
- ❖ To carry out deproteinization of serum.
- ❖ To demonstrate the creatinine-cobalt-2-NBA system for accurate electrochemical determination of serum creatinine.
- ❖ To designate all the redox peaks in the creatinine-cobalt-2-NBA system, supported by electrochemical and spectroscopic findings.
- ❖ To establish the dependence of the voltammogram response of aqueous copper sulphate solution on the direction of the potential sweep with bare platinum (Pt) electrode.

- ❖ To establish the impedimetric response of copper sulphate solution at different potentials.
- ❖ To study the creatinine-copper interaction in aqueous medium, from an electrochemical and spectroscopic perspective.
- ❖ To study the electrochemical response of creatinine with copper-deposited Pt electrodes.
- ❖ To fabricate a creatinine-imprinted-PPy-deposited Pt electrode and electrochemically corroborate the fabrication by recording voltammograms of copper sulphate solution and impedimetric responses of different concentrations of creatinine solution with the modified electrode.

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