NANOBIOSENSOR FOR RAPID DETECTION OF CREATININE CRYSTALS PRESENT IN URINE

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Abstract

Creatine is synthesized primarily in the liver and kidneys from amino acids and is phosphorylated to phosphocreatine, which serves as an energy source in muscle. Creatine is non-enzymatically degraded to creatinine at a constant rate and excreted in the urine. Its formation mainly depends on the muscle mass. Changes in creatine and creatinine levels may be related to various diseases (e.g. kidney disease, rhabdomyolysis etc.). Several methods are used to determine creatinine, such as for example the Jaffe method using the reaction of creatinine with picric acid in an alkaline medium, forming a red-orange complex or the enzymatic Trinder reaction. In some cases, crystals or larger aggregates are present in the urine. Primary diagnosis of such crystals is focused on microscopic evidence of the presence of the structure and may be confirmed by FTIR. The three enzymes, creatinase, creatininase and sarcosine oxidase can also be used for qualitative assessment of creatinine. The aim of this work was to design a SPION nanobiosensor for qualitative proof of the presence of creatinine in a sample. The methodology uses the highly sensitive Red Amplex fluorophore. Absorbance dependence on creatinine concentration read off at the wavelength of 572 nm was linear over a total range of 0 -1000 μM.

Keywords: Sarcosine; urinary crystals; photometric detection; automated analysis; sarcosine oxidase; SPION

1. INTRODUCTION

Creatinine is a metabolic product of creatine phosphate in the muscles, which provides energy to the muscle tissue. Creatinine levels are considered an indicator of renal function, thyroid disorders, and muscle damage. The serum creatinine level ranges between 45-140 μM and is excreted in the urine at 0.8 - 2.0 g/day [1]. The accuracy of serum creatinine concentration measurement affects the estimation of glomerular filtration rate (GFR) [2]. A simplified diagram of creatinine metabolism is shown in **Figure 1**. In addition to the above-mentioned importance of creatinine levels in clinical practice, there are more rarely occurring biologically significant phenomena. Elevated concentrations of low molecular weight substances can lead to the formation of small crystals to relatively large stones present in various organs. The analysis of urinary stones present in the urinary tract is of considerable diagnostic value and may provide significant benefits to the patient. Very few cases of creatinine/creatine crystals in urine have been described so far [3]. A number of analytical approaches (spectrophotometry, chromatography) are available for the determination of creatinine. In routine clinical practice, creatinine determination by non-enzymatic (Jaffé method) and enzymatic methods are used.

The principle of the Jaffé method is the reaction of creatinine with picric acid in an alkaline medium to form an orange-coloured complex. The enzymatic method is based on the conversion of creatinine by creatininase to creatine. Creatine is hydrolysed to sarcosine by creatinase. The sarcosine formed is oxidised by sarcosine oxidase to glycine, formaldehyde and hydrogen peroxide.

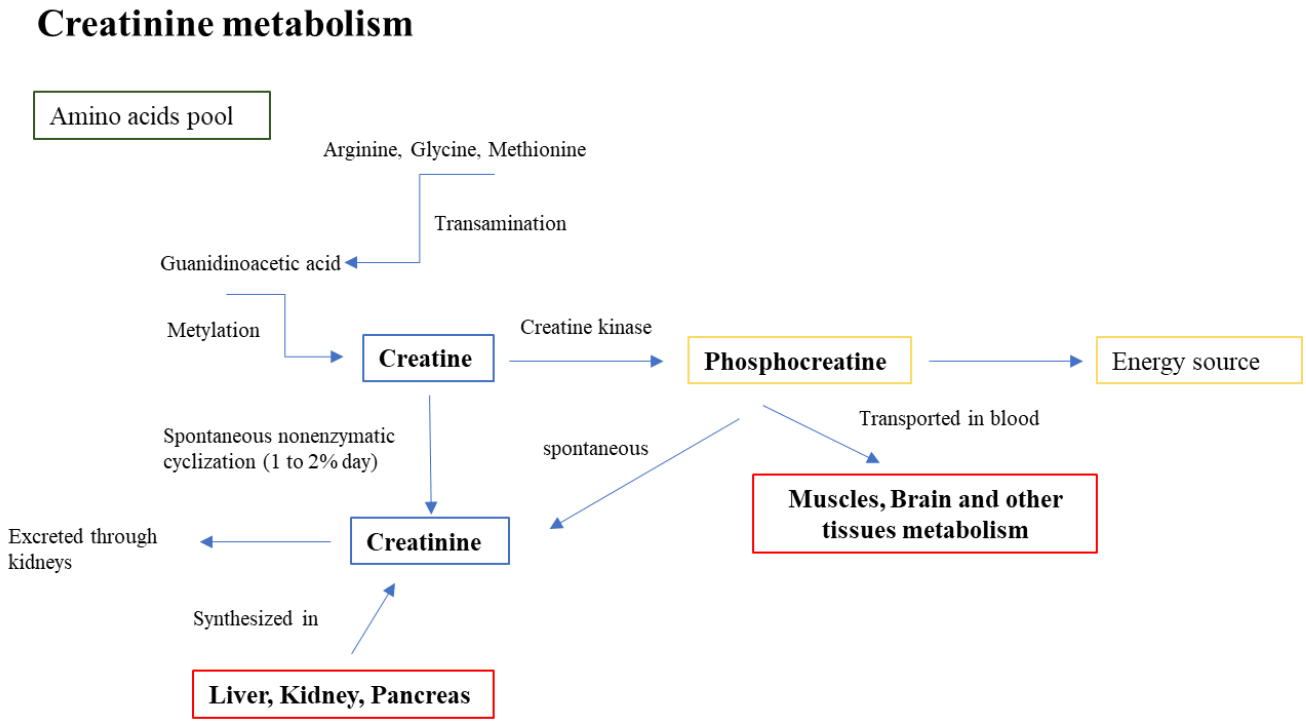

Figure 1 Simplified diagram of the proposed metabolic pathways of creatine and creatinine

It is well known that amino acids are colourless, making their direct photometric determination challenging. A suitable method for analysing amino acids involves their reaction with chromophores to form coloured complexes [4]. The well-known reaction of amino acids with ninhydrin produces a complex ranging from red-violet to blue-violet in colour [5], with the absorbance of these products measured at 570 nm [6-9].

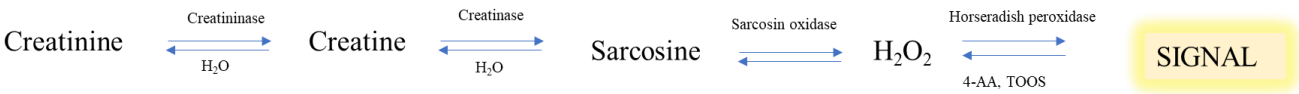

Figure 2 Simplified diagram of the proposed reactions enabling the measurement of creatinine concentration in a biological sample

Figure 2 illustrates a simplified scheme for the detection of creatinine. In the proposed method, creatinine is converted by creatininase into creatine. The subsequent reaction with creatinase leads to the synthesis of sarcosine. Sarcosine oxidase (EC 1.5.3.1) is a specific enzyme that oxidizes only the amino acid sarcosine in the presence of water and oxygen to glycine, producing hydrogen peroxide and formaldehyde as by-products. The resulting hydrogen peroxide can subsequently be quantified using the Trinder reaction, detectable spectrophotometrically at 510 nm. In this reaction, hydrogen peroxide interacts with phenol and 4-aminoantipyrine in the presence of the sodium salt of 3-(N-ethyl-3-methylaniline) propanesulfonic acid (TOPS) and peroxidase, leading to the formation of a quinonimine complex.

Biosensors, comprising a biologically active component, a chemical-physical transducer, and an advanced mathematical evaluation system, are also available and have demonstrated the capability to yield comparable

results. The potential of nanotechnology in bioanalytical approaches is substantial. This approach allows for significant enhancements in both the sensitivity and selectivity of analyses. Clinical laboratory medicine demands a high level of precision, often working with minimal sample volumes and requiring the avoidance of unnecessary repetition of tests. Nanotechnology presents a promising solution to these challenges. Numerous types of nanoparticles have been developed, with superparamagnetic iron oxide nanoparticles (SPIONs) being the preferred choice for efficient analyse detection. SPIONs can be easily modified with appropriate biomolecules, enabling the selective and sensitive detection of target analyses [16]. Consequently, this approach is increasingly integrated into rapid point-of-care testing (POCT) devices.

-The objective of this research is to design and develop an advanced nano-biosensor based on superparamagnetic iron oxide nanoparticles (SPIONs), specifically tailored for the precise and efficient detection of creatinine. This work places particular emphasis on refining in-house analytical methodologies to achieve high sensitivity and specificity in creatinine measurement. A critical issue addressed by this study is the current deficiency in rapid screening capabilities within point-of-care (POC) testing systems, which this novel SPION-based nano-biosensor is poised to significantly enhance.

2. MATERIAL AND METHODS

2.1 Material and chemicals

The analytical grade chemicals (ACS) used were purchased from Sigma Aldrich (USA): NaH_2PO_4, Na_2HPO_4, Na_3PO_4, KCl, NaCl, NaOH, HCl, 4-aminoantipyrine, phenol bioextra, 3-(N-ethyl-3-methylanilino)-propanesulfonic acid sodium salt, sarcosine, creatinine, bovine serum albumin, enzymes (horseradish peroxidase, sarcosine oxidase from Bacillus sp.). The disposable plastic tips and microtubes used were of RNA and DNA free quality (Eppendorf, Germany). Disposable 1 cm UV-VIS cuvettes for the single-beam spectrophotometer were purchased from VWR (USA).

2.2 UV-VIS spectroscopy

Spectrophotometric measurements were conducted using a UV-3100 PC spectrophotometer (VWR, USA). For these measurements, plastic cuvettes (UV-VIS, BRAND, USA) were utilized. 500 µL reagent was pipetted into the cuvette followed by 100 µL of sample. The reaction solution was used as blank. Further spectrophotometric analyses were performed on a polystyrene microtiter plate, which had been pre-washed with 18 MΩ water (3 times, 300 µL) at room temperature using a reader (TECAN, Switzerland). 50 µL of sample and 250 µL of reagent were pipetted. Absorbance measurements were taken every minute at 540 nm over a period of 60 minutes. The analysis was conducted using the VARIOSKAN Lux multimode reader (Thermo Scientific, USA). The scanning was performed across a wavelength range of 300 to 800 nm, with 1 nm increments. Data control and collection were managed using the SKANIT RE software (version 6.0.1).

2.3 Fourier-Transform Infrared (FTIR) Spectroscopy

All samples were dehumidified (ECOCELL BMT, Czechia, 130 ℃, 24 h) and maintained in a desiccator (silica gel) prior to analysis. The functional group modifications of the samples were characterized using Fourier-transform infrared spectroscopy (Nicolet iS10 Spectrometer) by the potassium bromide (KBr) pellet method. The dried samples (30 mg) were mixed with KBr (500 mg) and pressed under mechanical pressure in mortar (5 min). Spectra were recorded between the wavenumber range of 4000 and 400 cm^{-1}. Software was used to remove noise and smoothening utilizing Savitsky-Golay filter, baseline correction and Fourier transformation.

2.4 Automated analysis

For the chemical analysis of specimens, the Atellica Solutions CH 930 analyser (Siemens, USA) was used for photometric analyses. The urine sediment was analysed using Atellica 1500, which combines the Clinitek

Novus analyser and the Atellica Automated Urinalysis System 800 (Siemens, USA). The semi-automated Aution Eleven AE-4020 (Arkray, Japan) with Aution Sticks 10EA was utilized for the chemical urine analysis with a measurement principle of test strip method and dual-wavelength reflection photometric method. For the detection of ions, an integrated multisensor technology (IMT) on printed electrodes was employed. Osmolality was measured using the OsmoPRO osmometer (Advanced Instruments, USA).

2.5 Reaction procedure

Sample contained 4-AAP (4-aminoantipyrine), sarcosine oxidase, horseradish peroxidase in 0.2 M phosphate buffer (pH 8). After pipetting the sample into the reaction solution, incubation was performed for 30 minutes. Artificial urine: sodium chloride (170 mM), potassium chloride (80 mM), sodium phosphate (39 mM), urea (300 mM), creatinine (18 mM), bovine serum albumin (750 µM) were prepared according to available protocols [18-20].

2.6 Synthesis and modification of SPION

Preparation of SPION nanoparticles and their modification with SOX SPION nanoparticles were prepared according to the procedure published [21]. Shortly, SPIONs nanoparticles were prepared from $Fe(NO_3)_3$ and a solution of borohydride in 3.5% NH_3. The synthesis was carried out stirring at 100 °C for 2 hours. After 2 hours, the mixture was allowed to stir overnight at room temperature. The prepared iron nanoparticles were washed, and their surface was modified by the reaction with $HAuCl_4$, the mixture was stirred for 3 hours. Trisodium citrate was then added, and the mixture was stirred again overnight. The resulting SPIONs nanoparticles were washed, dried and ready for further use. For further modification, the SPIONs nanoparticles were weighed, washed and SOX was attached to them using chitosan and tripolyphosphate.

2.7 Microscopy

Microscopic analysis was performed on an OLYMPUS BX53 microscope paired with a CANON camera and an evaluation program (Quick PHOTO CAMERA 3.2). Dry urine sample (1 mg) was used on a glass slide after intensive grinding. For each magnification (100, 200, 400, and 600 times), ten images were evaluated in both transmitted and polarized lighting. Selected images were recorded in the evaluation program and saved.

2.8 Data treatment and descriptive statistics

The experimental work was carried out in three independent experiments. The analysis of each sample was carried out 5 times. The obtained data are presented as average values. From the proposed study no experimental subjects were excluded from the proposed experimental studies. All the obtained data were stored in the QINSLAB database (Czech Republic). When possible, data was processed and evaluated mathematically and statistically in the QINSLAB database. The exclusion of outliers in the data sets was performed by calculating the Grubbs test. Photographs were processed by programme ColorTest, which assigns intensity to the individual pixels of the studied image in the colour area. For the publication purpose, data were processed using Microsoft (USA).

3. RESULTS AND DISUSSION

The spectrophotometric analysis of biological samples conducted using automated techniques is illustrated in **Figure 3**. In the initial phase of this study, the effect of a substantial creatinine excess in a model serum sample was investigated. Our findings indicate that a creatine concentration of approximately 100 mmol/L induces a signal variation of up to 5%, thereby confirming the robustness of the proposed reaction mechanism (see **Figure 2**). It is well known that haemolysis can affect analytical accuracy, and this was assessed by testing haemolysis levels ranging from 0 to 6000 mg/L. As demonstrated, no significant dependence on haemolysis was observed. The method showed typical concentration-dependence relationships in urine (y = 40.1x - 45.2;

r = 0.9994, LOD = 2, LOQ = 6, QC = 3.4 mmol/L) and serum (y = 71.4x - 71.5; r = 0.9991, LOD = 1.2, LOQ = 3.8, QC = 4.2 µmol/L). Further evaluation of the method was performed using serum samples from 98 subjects, with creatinine concentrations ranging from 10 to 900 µmol/L. The data exhibited a normal distribution with a mean concentration of 174 µmol/L and a median of 91 µmol/L. In urine samples (n = 80), concentrations ranged from 700 to 30,000 mmol/L, displaying a normal distribution with a mean of 9872 mmol/L and a median of 8219 mmol/L. Additionally, this pilot study included a unique cohort of 64 children (age 0–1 year), with creatinine levels in serum ranging from 29 to 79 µmol/L, and a mean and median of 60 and 61 µmol/L, respectively.

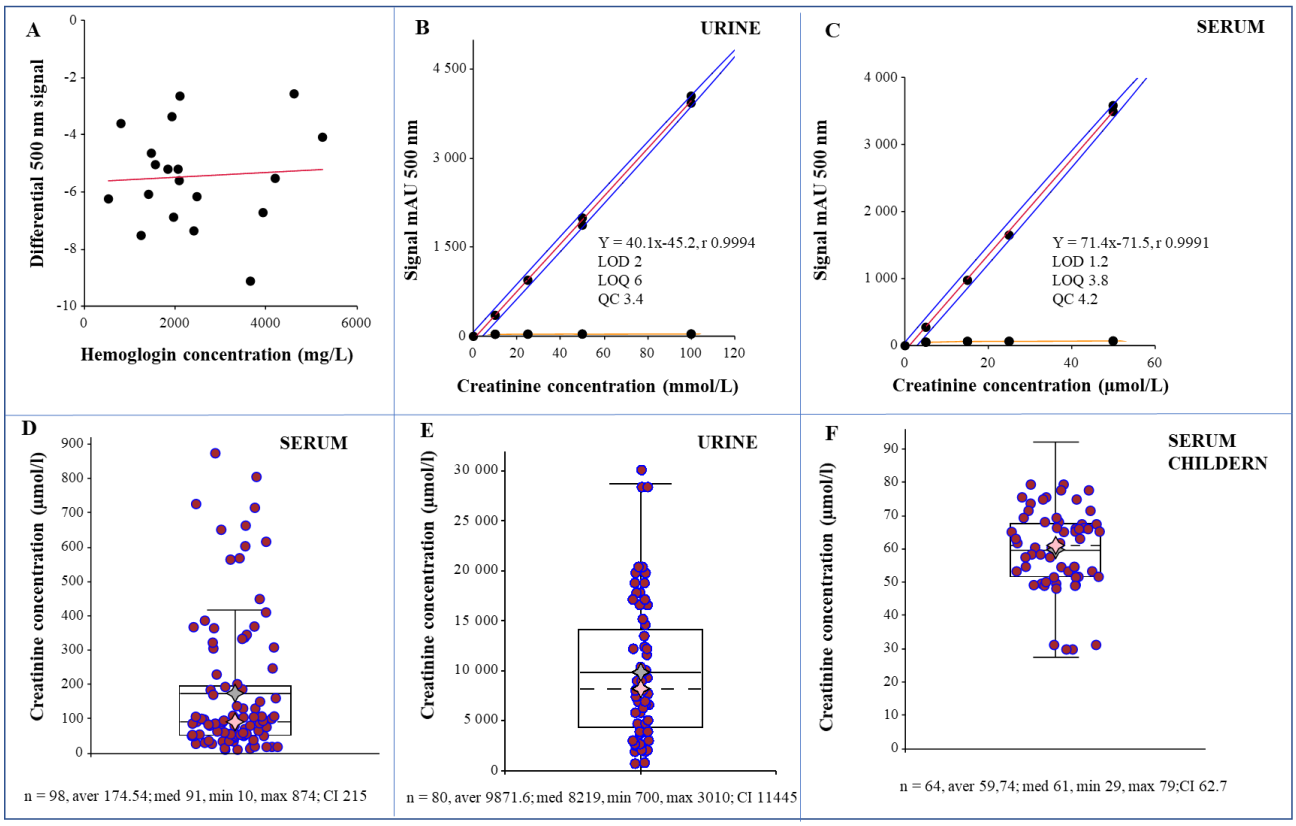

Figure 3 Spectrophotometric analysis of creatinine of biological samples by automated analyser. Study of the effect of erythrocyte haemolysis (haemoglobin 0 - 6000 mg/l) (**A)** on the measured signal of creatinine concentration in serum sample. Dependence of creatinine signal in model serum (0 - 60 µmol/l) (**B)** and in model urine (0 - 100 mmol/l) (**C).** Biological variability (including pathology) of serum creatinine concentration (n = 98) (**D)** and urine **(E)**, group of children (0 - 1 year; n = 64) (**F)**.

In our designed concept, chemical synthesis of magnetic particles (SPION) modified with citric acid and subsequently enzymes (creatininase, creatinase, sarcosine oxidase and peroxidase) was carried out. SPION modification was performed according to our previous work with chitosan [17]. Modification was carried out in 0.1 M phosphate buffer (pH 8.0) with added chitosan (8 mg/mL) for 30 min, 100 rpm, 25 C. After that, TPP (0.1 mg/mL) was added to the particles and further incubated for 30 min, 100 rpm, 25 C. Subsequently, the modified particles were washed 3 times (in 0.1 M phosphate buffer, pH 8.0). Based on the results obtained in **Figure 3**, we extended our approach to the analysis of other biological samples (blood, serum, urine, stones). The particles prepared in this way were used to detect the presence of creatinine (**Figure 4**). Attention was focused on the final stage of detection of sarcosine conversion by the enzyme sarcosine oxidase. In the experiment, creatinine was determined by an enzymatic method using 4-aminoantipyrine (4-AAP) and the sodium salt of 3-(N-ethyl-3-methylaniline) propanesulfonic acid (TOPS). The use of these reagents produces a stable mixture

with very good analyte sensitivity. Creatinine is converted by creatininase into creatine and the reaction with creatinase leads to the synthesis of sarcosine. Sarcosine oxidase oxidizes sarcosine in the presence of water and oxygen to glycine, producing hydrogen peroxide and formaldehyde as by-products (see **Figure 2**). The product of the reaction is a distinct purple coloured quinonimine complex with an absorption maximum at 546 nm, as shown in **Figure 4C**. Very good responses were obtained in the tested samples even at low creatinine concentrations. The procedure is therefore suitable for further development of POCT technologies.

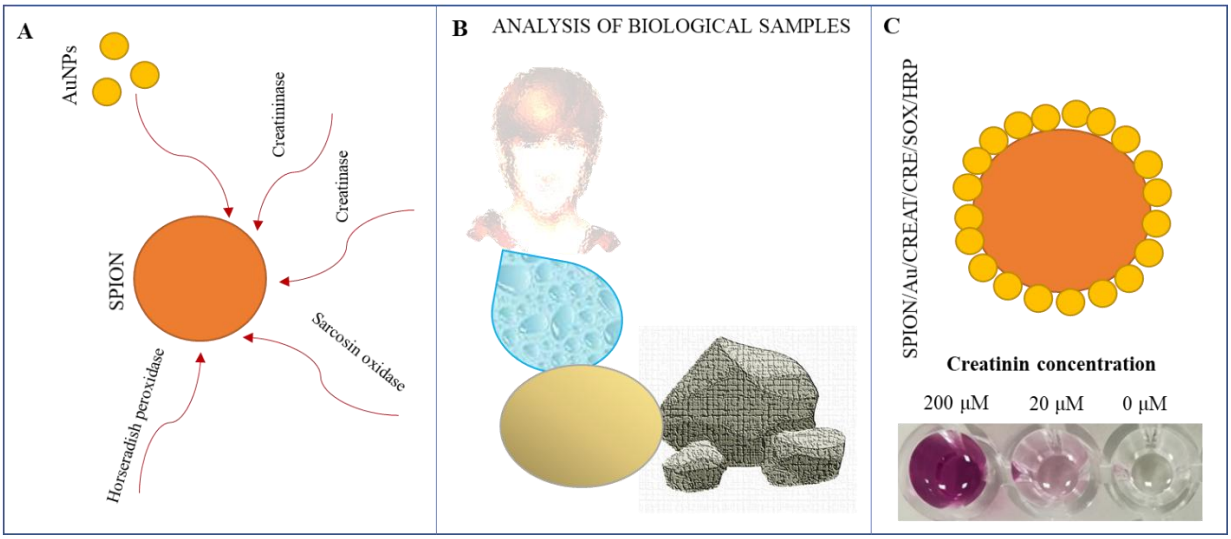

Figure 4 Scheme of SPION modification with gold nanoparticles AuNPs and enzymes sarcosine oxidase, creatinase, creatininase (**A**). Potential use of SPION/Au/CREAT/CRE/SOX/HRP for biological sample analysis (serum, blood, urine, exudates, concrements) (**B**). Color reaction of detected creatinine (200, 20, 0 µM) in 1 mM PBS using modified nanoparticles (**C**)

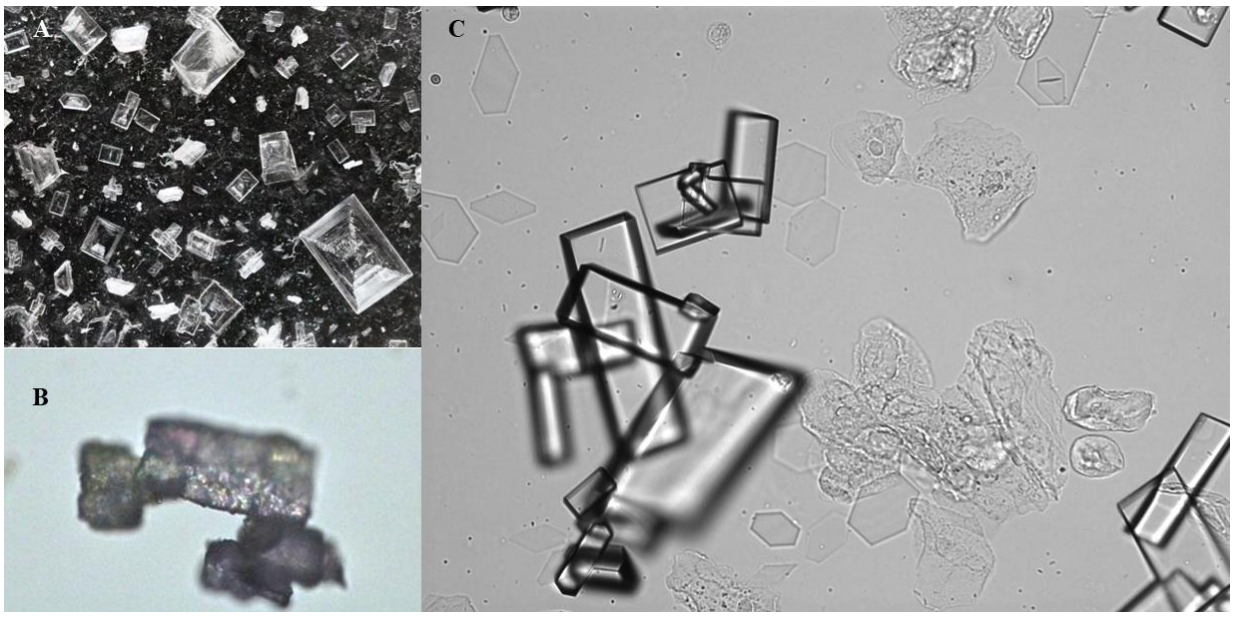

Figure 5 Crystallisation of creatinine (**A**). Polarization microscopy of creatinine crystals (**B**). Light field microscopy of creatinine crystals in a biological urine sample (**C**)

The present study focuses on the application of the proposed concept to the analysis of unique samples, such as crystals. Although it is exceedingly uncommon for creatinine/creatine crystals to manifest, with the majority

of cases occurring within the urinary tract [22], recently, we were able to document a case of this phenomenon. By employing a range of analytical techniques, we were able to confirm the presence of these crystals. However, it remains uncertain whether this infrequent occurrence is attributable to the specialisation of the department and whether the requisite tools are accessible in less specialised departments. This hypothesis may be verified by the modified SPIONs developed by our research group. Moreover, our team has also synthesised creatinine and creatine crystals. The structure of creatinine crystals was first elucidated over seven decades ago [23], and it is established that diverse complexes can be formed [24]. The resulting crystals are illustrated in **Figure 5** and were subsequently subjected to a comprehensive analysis. The presence of creatinine was confirmed in the samples using the specified analytical techniques.

4. CONCLUSION

In routine laboratory operations, the limited space and time prevents a more detailed study and analysis of the biological sample. Usually, a rapid and one-time examination is necessary, which is an approach not sufficiently sensitive to detect rare occurrences, such as the presence of creatinine crystals. Our approach involved the design of a rapid procedure for specific identification, which was made possible by the use of nanotechnology. SPION nanoparticles were modified with enzymes for this purpose. This resulted in the development of a highly sensitive and selective tool that is suitable for incorporation into a point-of-care testing (POCT) analyser.

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