Towards Sarcosine Determination in Urine for Prostatic Carcinoma Detection

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Abstract.

Sarcosine, a potential biomarker for prostate cancer, can be detected in a solid state enzyme based biosensor using sarcosine oxidase, with particle immobilised reagents. A novel fusion protein of the fluorescent protein, mCherry, sarcosine oxidase (SOx), and the polypeptide R5 ($R5_2$-mCherry-SOx-R5-6H), was explored, which allowed self-immobilization on silica microparticles and long-term (90 days +) retention of activity, even at room temperature. In contrast, commercial wildtype SOx lost activity in a few days. A silica-$R5_2$-mCherry-SOx-R5-6H microparticle sensor for determination of sarcosine in urine, linked the SOx coproduct, H_2O_2, to a measurement catalysed by horseradish peroxidase (HRP) immobilised on silica, in the presence of Amplex Ultrared (AR) to generate fluorescence at 582 nm. Silica microparticles carrying all the reagents ($R5_2$-mCherry-SOx-R5-6H, HRP and AR) were used to produce a silica-microparticle biosensor which responded to sarcosine at micromolar levels. Interference by amino acids and uric acid was examined and it was found that the silica-reagent carrying system could be calibrated in urine and responded across the clinically relevant concentration range. This contrasted with similar assays using commercial SOx, where interference inhibited the sarcosine signal measurement in urine. The microparticle biosensor was tested in urine from healthy volunteers and prostate cancer patients, showing higher concentrations of sarcosine in cancer patients consistent with previous reports of elevated sarcosine levels.

Keywords Silaffins, Enzyme immobilization, Biosensor, Sarcosine, Prostatic carcinoma, urine

1. Introduction

Development of in vitro diagnostics (IVDs) to guide earlier diagnosis and more effective

clinical intervention with improved cost effectiveness has become a clear objective in sensor research [1]. Cancer is not yet well represented in IVD diagnostics, but the need for early detection and triage is undisputed. It has been noted by Soper et al. [2] that point of care diagnostics for cancer have been slow to evolve for a variety of reasons. In some cancers this is partly due to the lack of known robust biomarkers that could act as targets in a diagnostic. In other cancers, some progress could be made with the production of the right reagents for identified biomarkers.

Prostate cancer, for example, is one of the leading causes of cancer death among men [3]. Although widely used in connection with cancer, detection of elevated levels of prostate-specific antigen (PSA) will identify prostate disease, but not necessarily prostate cancer. When the test was first introduced in 1986, it was intended as a marker of disease progression, in already diagnosed patients, not for cancer diagnosis, but its wider use [4] and the resulting ambiguity in the result is now driving the search for better biomarkers and thence IVDs [5-7]. Thus, biopsy and digital rectal examination (DRE) are still the best first approach [8] in combination with PSA testing. Centralised laboratory testing further supports diagnosis with, for example, transrectal ultrasound [9] guiding biopsies [10] and an increasing use of magnetic resonance [11] and positron emission tomography [12].

PSA is typically measured in blood or serum (recently a study in saliva has also been reported [13]), but *to add value* to the PSA test, urine based biomarkers are of special interest, that can be 'co-detected' without invasive sampling, as described by Cao et al [7]. Molecular biomarkers (metabolite, gene and protein based) are being identified that collectively may lead to better diagnostics [14, 15] and, in this context, the amino acid, sarcosine (N-methylglycine) has been studied in both blood and in urine as a marker of early-stage prostate cancer [16]. Although the measurements in blood have been reported as unremarkable, significantly elevated levels in urine have been recorded in some studies, for patients with prostate cancer. Furthermore, in a recent study cancer sniffer dogs were able to distinguish artificial urine samples that had been doped with sarcosine, with 90% success rate [17]. The possible role of sarcosine is however still strongly debated [18], but some recent studies have suggested that sarcosine exhibits considerable stimulatory effects on growth in malignant/metastatic prostate cells [19], possibly due to accumulation in the tumor and consequential conversion to serine and glycine, thereby providing tumor growth promoters [20,21].

Laboratory based techniques for sarcosine detection are typically high performance chromatography [22], whereas several different approaches have been suggested to provide a nearer-patient diagnostic. For example, Biavardi et al., [23] designed a direct binding cavitand,

anchored on a Si-substrate, that showed a change in the fluorescence in the presence of sarcosine, whereas Valenti et al., [24] used the same cavitand to provide selectivity in an electrochemiluminesce system. In contrast, others have proposed an immunoassay [25] which was able to discriminate sarcosine in urine samples with good sensitivity and without undesired interference in well controlled assay conditions, although quantification in a near-patient diagnostic measurement remains challenging.

Currently available sarcosine test *kits* mainly use a classical oxidase linked assay, and although there are reports of amperometric sarcosine biosensors with sarcosine oxidase, using the same principle as first and second generation glucose oxidase biosensors [26], most of the test kits that are already available are colorimetric or fluorometic versions of this classical oxidase assay. These are based on an indirect measurement of sarcosine via the coproduct H_2O_2, catalysed by horseradish peroxidase and taking advantage of the myriad of dyes that can be coupled into this reaction as indicator. Nevertheless, although these types of kit are suitable for use in plasma and serum, interference in measurements made in urine is typically reported, producing erroneous results. Some attention has been given to overcoming this interference, for example Lan et al. used 3,3',5,5'-tetramethylbenzidine with palladium nanoparticles as a catalyst instead of horseradish peroxidase [27] and Burton et al. directed their attention to the other coproduct (formaldehyde) and the pH change associated with its conversion to formic acid under optimised conditions [28]. However, these methods have required quite a lot of laboratory based processing in the analytical pathway; for example the latter method [28] requires the introduction of NaOH and heat, for the Cannizzaro reaction to proceed which converts the formaldehyde to formic acid and methanol and degrades the hydrogen peroxide. This isn't suitable for development of a biosensor and thus, interference of the measurement in urine remains a challenge for a near patient diagnostic.

Most of these assays use solution based reagents. However, it has been reported that immobilized enzymes can in some cases, exhibit higher selectivity and sensitivity [29], but on the other hand, chemical modification during immobilization can cause enzyme degradation or block the active centre, or result in the enzyme orientation being incorrect for reaction with the enzyme substrate. [30] Peptide molecular biology immobilization techniques can be inspired by high affinity peptides from binding patterns in nature [31] which allows an immobilisation functionality to be fused with the reagent enzyme (e.g. sarcosine oxidase) during protein expression. This allows attachment of the protein on a surface to be in-built without further chemical modification and may help to better retain enzyme activity. We have recently taken inspiration from "Garage Biotech" [32] and shown that an engineered sarcosine oxidase (SOx), immobilised on silica via a silaffin peptide tag [33] can retain exquisite selectivity for sarcosine.

A simple one-step isolation-to-use method is presented which produces a silica particle-enzyme sensor. It is low cost, biocompatible and stable in most biosystems [34].

We have therefore returned to the sarcosine oxidase linked assay and considered whether the assay can be optimised for measurement in urine by focusing on the presentation of the enzyme and other reagents, and thereby, develop a biosensor where all the reagents are immobilized on a solid support, leading to good point of care usability, rapid result outcome. Successful (bio)molecule immobilization has been demonstrated to be beneficial for diverse biotechnological and medicinal applications [35]. In the research reported herein, we use both chemical immobilisation techniques and protein engineering to improve long term protein stability and aid performance in the design of in vitro diagnostics (IVD). We used the novel enzyme-silica combination in order to study its compatibility in urine and to develop a fluorometric sensor for sarcosine detection in human urine, by incorporating all needed reagents in silica. A first small sample test shows discrimination between healthy and prostate cancer volunteers.

2. Materials and methods

2.1 Materials and reagents

Enzymes used for cloning (restriction enzymes, calf intestinal alkaline phosphatase, Antarctic phosphatase, T4 DNA ligase) and Quick Ligation kit were purchased from New England Biolabs and were used according to the manufacturer's instructions. Plasmid pET24a (Novagen) containing the $R5_2$-mCherry-mSOx-R5-6H gene was produced previously in the Hall laboratory, Cambridge. Details have been reported previously [33].

The enzyme sarcosine oxidase from Bacillus sp. (25-50 units/mg), peroxidase from horseradish (50-150 units/mg), lysozyme from chicken egg white lyophilized powder and the analyte sarcosine were purchased from Sigma Aldrich (Saint Louis, USA). The particles used were glass bubbles microspheres/microballoons from easycomposites (Stoke-on-Trent, UK), gel silica particles <63 μm from Fluka (Monte Carlo, Monaco), TLC-Kieselgel 60H average particle size 15 μm (90% between 3.5 – 25μm) from Merck (Darmstadt, Germany) and microparticles from YMC (Hong Kong, China) (range 63-210μm) and glass beads unwashed (450-600 μm) were from Sigma Aldrich (Saint Louis, USA). The silica particles were modified with tetraethyl orthosilicate, 3-(aminopropyl)triethoxysilane ammonium hydroxide solution (28-30%) from Sigma Aldrich (Saint Louis, USA). The fluorescent probe Amplex Red was

obtained from Sigma Aldrich and the sensitive Amplex UltraRed was provided by Invitrogen, ThermoFisher Scientific.

The artificial urine was prepared using urea, creatinine, sodium chloride, sodium bicarbonate, calcium chloride, sodium phosphate monobasic, citric acid provide from Sigma Aldrich (Saint Louis, USA) and ammonium sulphate and sodium sulphate obtained from Fischer Scientific (Waltham, USA). Arginine, tryptophan, serine, glycine leucine and uric acid were used to test the selectivity of the assay and were purchased from Sigma Aldrich (Saint Louis, USA). Urine samples were provided by the La Fe Hospital and Clínico Hospital of Valencia, Spain. Dark 96-well plates and clear 96-well plates used for fluorescence and absorbance measurements respectively, were provided by Thermo Scientific (Roskilde, Denmark).

2.2 Instruments

A Varian Cary Fluorescence Spectrophotometer from Agilent (Santa Clara, USA) was used for fluorescence measurements and a spectrophotometer synergy HT from Biotek Intruments Ltd (Winooski, USA) was used for absorbance measurements. Characterization of particles by FTIR-ATR was carried out with a Cary 630 FTIR-ATR spectrophotometer Agilent Technologies (Böblingen, Germany). A Nikon EFD-3 microscope was used for the optical and fluorescence images.

The incubator used for the synthesis of the fusion protein was the Innova 4300 incubator shaker from New Brunswick Scientific (Enfiel, USA). The centrifuge used was megafuge 1OR for larger volume (flacon tubes) and Biofuge pico for small volume (eppendorf) both form Heraeus Intruments (Hanau, Germany). The pH values were determined with pH meter 3510 from Jenway (Staffordshire, UK) calibrated at room temperature with standard buffers.

2.3 Expression and isolation of the protein

Protein design, expression and isolation has been described previously [33]. The constructs were designed with a coloured fluorescent protein (mCherry, mCh) to enable easy monitoring of production, and a silaffin peptide sequence for immobilisation to silica. The plasmid was designed previously and provided for this project. Before expressing the protein, BL21(DE3) E. coli containing the desired plasmid were grown overnight in a starter culture of 20 ml Luria Broth (LB). Then, 200 µL from overnight culture was transferred to 20 mL of LB Broth with kanamycin (10 µL, 100 mg/mL) and the solution was left overnight in the 37ºC shaking incubator. The culture was transferred to 200 mL LB Broth with kanamycin (100 µL, 100

mg/mL) and it was incubated for 3 h in the shaking incubator. Then, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added as initiator and it was incubated for 4-5 h during protein expression. Due to the internally fused mCherry label, production could be followed by the development of the pink colour. The culture was centrifuged at 4300 rpm for 20 min at 4ºC. The supernatant was discarded and the pellet was stored at 4ºC. The protein was obtained from the cell by lysing with lysozyme (10 mg/mL), sonication for 30 s on /30 s off for 3 cycles; 20 s on / 30 s off for 5 cycles; 10 s / on 10 s off for 10 cycles. The mixture was centrifuged at 13000 rpm for 20 min at 4ºC, the supernatant which contained the impure protein was stored at 4ºC.

2.4 Immobilization on silica particles

The immobilization of $R5_2$-mCherry-mSOx-R5-6H on silica particles followed the procedure reported previously [33] with some modifications. The modified protocol was carried out using a suspension of silica particles (10 mg/mL, 5 mg in 0.5 mL), prepared in disodium phosphate buffered saline (100 mM phosphate, 150 mM NaCl, pH 7.5). The mixture was sonicated for 1 h and then 150 μL of crude protein (0.8 mg/mL) was added. The final concentration of the protein was 0.18 mg/mL. After mixing it by vortex, the suspension was left for 1 h at room temperature to precipitate. The particles became pink and the supernatant colourless. Finally, it was centrifuged at 13000 rpm for 5 min. The final protein loading for SiO_2-$R5_2$-mCherry-mSOx-R5-6H was 24μg protein/mg silica.

The selectivity of $R5_2$-mCherry-mSOx-R5-6H for silica against other materials such as alumina, titanium oxide, calcium carbonate, cellulose and chitosan and were tested following the same protocol. Also, the protocol was followed to test the immobilization of $R5_2$-mCherry-mSOx-R5-6H for different particle size of silica.

To test the $R5_2$-mCherry-mSOx-R5-6H loading on silica, different concentration of crude protein (0.8mg/mL) or pure protein (4mg/mL) were added to 5 mg of silica in 0.5 mL of disodium phosphate buffer saline (100mM phosphate, 150 mM NaCl pH: 7.4) separately and the protocol was followed.

To evaluate the $R5_2$-mCherry-mSOx-R5-6H resistance to leaching: the particles were washed several times with water and buffer and no leaching of the protein from the particles to the solution was observed. The R5-protein could be partially released in acidic media (pH 4), after 30 min (around 40% of release) while higher release (85%) could be achieved using lysine solution 1M, pH 7.4.

For the chemical immobilization of commercially available SOx and HRP, the protocol followed was developed previously by the Cambridge Analytical Biotechnology group for protein labelling of silica nanoparticles reported by [36,37]. The modified protocol described here, the NH_2-modified silica particles were obtained by mixing TEOS (0.6 mL) in an ammonia solution (30 % wt, 0.5 mL) and 0.1 g of silica particles. The mixture was stirred for 1 h. then, APTES (0.2 mL) was added and the mixture was agitated for 4-5 h at room temperature. After the reaction was completed, the particles were centrifuged, washed several times with water and ethanol and were dried overnight. Finally, a glutaraldehyde (GA) aqueous solution (1.6 % wt GA, 1 mL) was added to the obtained GA modified silica particles and the reaction was stirred for 3 h at room temperature. The particles changed from white to yellow and then, orange. The GA-silica particles were centrifuged, washed several times with water. Finally, they were dispersed in a solution of HRP (2 mg/mL, 1mL giving 0.04 HRP/2mg particles) or SOx (1.5 mg/mL) and stirred for 4-5 hours at room temperature. The HRP-SiO_2Ps and SOx-SiO_2Ps were centrifuged and washed three-four times with water and dried overnight.

Amplex Ultrared was immobilized by mixing TEOS (0.6 mL) in an ammonia solution (30 % wt, 0.5 mL) and 0.1g of silica particles with stirring for 1 h. 20 µL of stock solution of Amplex Ultrared in DMSO (10 mg/mL) was then added and stirred for 30 minutes. Finally, the supernatant was removed by decantation and the particles were dried at room temperature in the dark for 4 h. The colour of the particles changed from white to pink. The same experimental process was used for the immobilization of Amplex Red.

2.5 Estimation of immobilized protein on silica particles

Protein loading was checked by comparing data from two methods. Taking advantage of the fluorescence of the fused internal mCherry reference, direct estimation of immobilized protein is possible. The percentage of immobilized protein was calculated by the difference of fluorescence intensity ($\lambda_{ex/em}$ 587 / 607 nm) of the free protein in the supernatant at the beginning (I_{sol}) and end (I_{sup}) of the immobilisation, in equation (eq.) 1 :

$$\%\ of\ immobilized\ protein\ on\ silica\ = 100 - \left(\frac{I_{sol} - I_{sup}}{I_{sol}} \cdot\ 100\right) \qquad \text{(eq. 1)}$$

The Bradford assay [38] for the estimation of protein concentration using Coomassie brilliant blue and bovine serum albumin as a standard was employed.

2.6 Estimation of the enzyme activity

Trinder's colorimetric assay was adapted for sarcosine oxidase and used to measure the activity of $R5_2$-mCherry-mSOx-R5-6H in solution and immobilised on SiO_2 particles compared with commercial sarcosine oxidase. The assay was carried out in disodium phosphate buffer (150-300 μL, 10 mM, pH 7.5) with 4-aminoantipyrine (50 μL, 1% w/v), phenol (50 μL, 1% w/v), sarcosine (50 μL, 2 M), HRP (50 μL, 0.4 mg/mL) and the $R5_2$-mCherry-SOx-R5-6H (10 μL, 4 mg/mL) or SiO_2-$R5_2$-mCherry-SOx-R5-6H (2.5 mg, 4 mg/mL) or commercial sarcosine oxidase (10 μL, 2.4 mg/mL). After 5 min, 2.5 mL of ethanol was added to stop the reaction and the absorbance was registered at 480 nm. Total protein was measured by Bradford assay [42]. Specific activity was estimated by calculating the Units of enzyme activity per mg of total protein measured.

A similar protocol was used to measure the activity of HPR and HRP-SiO_2 particles. A solution of disodium phosphate buffer (300 μL, 10 mM, pH 7.5) with 4-aminoantipyrine (50 μL, 1% w/v), phenol (50 μL, 1% w/v), H_2O_2 (50 μl, 2M) and the HRP (10 μL, 0.4 mg/mL) or 2 mg of HRP-SiO_2Ps (containing 40μg protein). After 5 min, 2.5 mL of ethanol was added to stop the reaction and the absorbance was registered at 480 nm.

2.7 Sarcosine measurements

Preparation of artificial urine. Minimal artificial urine has been constituted for different purposes [39]. In this case artificial urine was constituted based on the Brooks and Keevil recipe, adjusted to be protein-free at pH = 6.2 and comprising of urea (170 mM), ammonium sulphate (12.5 mM), sodium chloride (90 mM), sodium bicarbonate (25 mM), sodium sulfate (10 mM), creatinine (5.30 mM), calcium chloride (2.5 mM) sodium phosphate monobasic anhydrous (7 mM), citric acid (2 mM). The solution was stored in the refrigerator (4 ºC) until use.

Determination of sarcosine using the reagents in solution. The determination of the sarcosine was based an enzyme linked assay using sarcosine oxidase and the indirect detection of H_2O_2, using Amplex Ultrared (AR) and horseradish peroxidase (HRP) as a catalyst (See Fig. 1B). Different volumes of sarcosine solution (0-10 μL, 100 μM) were added to 70 μL artificial urine or urine, followed by 10 μL of HRP 0.4 mg/mL and 10 μL of SOx 1.2 mg/mL and 10 μL of AR (100 μM) to a final volume of 100 μL. After reaction for 20 min at room temperature in the dark, the fluorescence was measured ($\lambda_{ex/em}$ 530 / 582 nm). The increment of fluorescence intensity of sarcosine standard (I_{Sar}) with respect to the blank (I_0) was plotted against the

concentration of sarcosine and expressed as follows: $I_{stand} - I_0 = a + b$ [Sarcosine]

Determination of sarcosine using the immobilized reagents on silica particles. Different volumes of sarcosine solution (0-10 µL, 100 µM) were added to a final volume of 100 µL of artificial urine containing 5 mg of protein-SiO_2 particles, 2 mg of HRP-SiO_2 particles and 5 mg of AR-SiO_2 particles. After reaction for 20 min at room temperature in the dark, the fluorescence was measured ($\lambda_{ex/em}$ 530 / 582 nm) as above. The same experimental process was followed in urine to obtain the calibration curve for sarcosine standards in control urine.

Well-cards were produced a 5mm diameter wells, cut from 1mm PMMA and sealed on one side with PCR plate seal (Thermo Scientific Adhesive PCR Plate Seal). 4 µL each of HRP (0.4 mg/mL), sarcosine (0-5µM final concentration) and AR (10µM) were added to 125µg silica-$R5_2$-mCherry-mSOx-R5-6H suspended in artificial urine (20µL total assay volume). Fluorescence intensity was recorded with an FFEI reader (505nm LED for excitation and a CCD RGB linear sensor for detection). Rate of change of average intensity in the well was plotted against final concentration of sarcosine, blank subtracted.

The design and construction of the hourglass sensors has been described in detail elsewhere [33] for the protein modified silica. They were produced by laser cutting the hourglass shape from 2mm PMMA and sealing both sides with PCR plate seal (Thermo Scientific Adhesive PCR Plate Seal). A suspension (total 220µL) of the $R5_2$-mCherry-mSOx-R5 immobilized on silica (1 mg silica, 0.025 mg/mL protein per 1mg silica) was mixed with AR (10µL, 100µM), HRP (10µL, 0.4mg/mL) and sarcosine (final concentrations 0µM - 11.75µM)) in artificial urine /urine was loaded into the device. The device was inverted twice for mixing, each time allowing the particles to settle to the bottom (60s). Fluorescence intensity was recorded with an FFEI reader (505nm LED for excitation and a CCD RGB linear sensor for detection) every minute for 18min. The images were analysed for grey scale intensity of the red channel in the detection area using ImageJ software.

Preliminary analysis of urine samples. Samples from 5 healthy volunteers and 10 cancer patients were provided from Hospital la Fe and the Clinic Hospital, Valencia, Spain and were stored at -20ºC until testing. The samples were brought to room temperature for 10 min and used for the assay without treatment or dilution. For the assay, 100 µL of urine containing 5 mg of protein-SiO_2 particles (containing <120µg protein), 2 mg of HRP-SiO_2 particles (containing <40µg protein) and 5 mg of AR-SiO_2 particles. After reaction for 20 min at room temperature in the dark, the fluorescence was measured at ($\lambda_{ex/em}$ 530 / 582 nm). The increment of fluorescence intensity of sarcosine standard (I_{Sar}) with respect to the blank; real urine with a sarcosine content

below LOD, (I_0), for each sample was interpolated on the calibration curve of sarcosine standards in urine in order to obtain concentration of sarcosine.

2.8 Selectivity and stability

For the selectivity, stock solutions of amino acids such as alanine, arginine, glycine, leucine, serine and tryptophan and uric acid were prepared at concentration of 1 mM. The solutions were diluted in artificial urine to final concentrations of 10 µM. A mixture of all of them with sarcosine were prepared to final concentrations of 10 µM. In addition, a sarcosine standard of 10 µM in artificial urine (I_{Sar}) and a blank (I_0) was used as a reference. Then, 5 mg of protein-SiO_2Ps, 2 mg of HRP-SiO_2Ps and 5 mg of AR-SiO_2Ps were added to 100 µL of each diluted solutions (I_i) and after reaction for 20 min at room temperature in the dark, the fluorescence was measured at excitation/ emission wavelength 530 / 582 nm. The relative fluorescence was calculated as follows:

$$Relative\ fluorescence\ (\%) = \frac{I_i - I_0}{I_{Sar} - I_0} \cdot 100 \qquad \text{(eq. 2)}$$

For the stability of the mSOx, a solution of commercial SOx (2.4 mg/mL) was stored for 90 days at room temperature. The obtained $R5_2$-mCherry-SOx-R5-6H and the immobilized protein on silica were also stored at room temperature for 90 days. A fresh solution of commercial SOx at 2.4 mg/mL prepared before the analysis as well as just expressed protein and immobilized on silica were used as a reference. A volume of 10 µL of fresh ($I_{i\ 0\ day}$) and stored solutions ($I_{i\ 90\ days}$) of the commercial SOx, $R5_2$-mCherry-SOx-R5-6H or 5 mg of SiO_2-$R5_2$-mCherry-SOx-R5-6H were dispersed in artificial urine. Then, 10 µL of sarcosine standard (100 µM) or 10 µL of water for the blank (I_0), 10 µL of HRP 0.4 mg/mL and 10 µL Amplex Ultrared were added. The final volume of each one was 100 µL. After reaction for 20 min at room temperature in dark, the fluorescence was measured at excitation/ emission wavelength 530 / 582 nm. The relative fluorescence was calculated as follows:

$$Relative\ fluorescence\ (\%) = \frac{I_{i\ 0\ day} - I_0}{I_{i\ 90\ days} - I_0} \cdot 100 \qquad \text{(eq. 3)}$$

For the stability of the fluorescence of mCherry-6H and R5-mCherry-6H on thin-layer chromatography (TLC) was measured by deposition of 1µL of each (1mg/mL) on silica TLC plates using glass capillaries. Then, the plates were left at room temperature for 1 month. Images of the spot were taken under the fluorescence microscope and fluorescence scans were performed with an FFEI reader (505nm LED for excitation and a CCD RGB linear sensor for

detection) at various time points. Fluorescence intensity in the red channel of the scans was quantified using ImageJ.

3. Results and discussion

3.1 Components for a silica-SOx biosensor platform.

Sarcosine oxidase belongs to the same family of enzymes as the ubiquitous glucose oxidase, so that in considering an approach to sarcosine measurement in urine, many lessons can be learned from the long history of glucose oxidase biosensors. However, in comparison with the two electron oxidation mechanism involving glucose oxidase [40], which has a specific activity ranging from 172-300 U/mg (μmol/min/mg), the specific activity for the one electron process with sarcosine oxidase is more than an order of magnitude lower. This poses significantly greater challenges in localizing sufficient enzyme in the vicinity of a transducer to generate a measurable signal for a biosensor and retaining sufficient activity in the immobilisation process. Using the classical glutaraldehyde coupling method for protein crosslinking, the specific activity of sarcosine oxidase (SOx) is reduced from ~9U/mg to ~0.09U/mg. This can be improved by chemical coupling with an NH_2 activated surface on silica particles, but the residual activity remains too low (~0.4U/mg, table 1) for use in a biosensor. In this work therefore, we consider self-immobilization directly from the protein without chemical coupling, by using synthetic biology to design a multicomponent fused protein. The philosophy of this approach is to include capacity for selective immobilization in the original enzyme design, by fusing an affinity peptide to the enzyme prior to expression.

Many affinity peptides have been identified for different materials [31] and in this example we have considered the use of silica as the core material platform for enzyme presentation. We have previously expressed a novel fusion protein: $(R5)_2$-mCherry-sarcosine oxidase-R5-6H ($R5_2$-mCherry-SOx-R5-6H) [33]. The R5 peptide described here is a 19 amino acid peptide (NH_2-SSKKSGSYSGSKGSKLLIL-COOH). It is derived from diatoms [41, 42] and enables the protein to "self-immobilise" by adsorption on silica particles (See Fig. 1). It has previously been reported in protein encapsulation with silica [43] or to enhance precipitation of the protein through silica encapsulation [44]. In contrast, we do not seek encapsulation, but have selected this peptide as an affinity tag for immobilisation on silica substrates. Leading on from preliminary results [33], the application of this silica-immobilised sarcosine oxidase is now explored in determination of sarcosine together with HRP and Amplex Red as silica bound

reagents for a solid state measurement in urine, without the interference that is typical when this assay is deployed in this sample matrix.

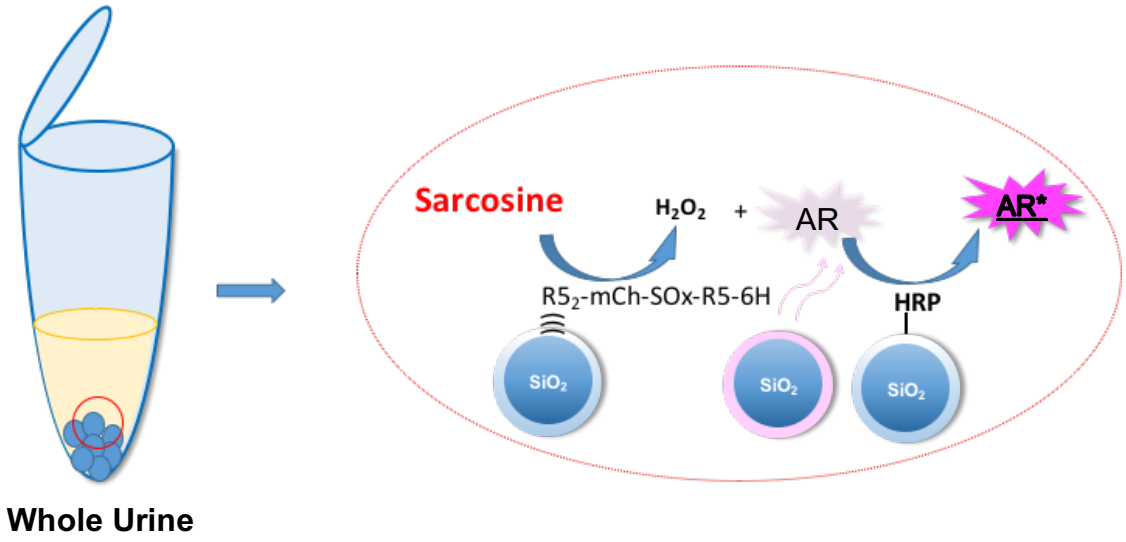

Fig. 1. Scheme of the biosensor platform for the assay of sarcosine determination in urine. $(R5)_2$-mCherry-Sarcosine oxidase-R5-6H, the catalyst horseradish peroxidase (HRP) and the fluorophore Amplex Ultrared (AR, pink surface on silica particle) were immobilized on silica particles. The sarcosine oxidase generates H_2O_2 in the presence of sarcosine which in turn results in the conversion of AR to a fluorescent product by HRP.

A fluorometric assay for sarcosine has been proposed based on the pathway reported in Fig.1. Central to the design of this system is the role of the R5 peptide on protein immobilization on silica. The experimental process of R5-protein immobilization is an easy, fast method where no additional coupling reagents are needed, other than the protein and the silica substrate in buffer. The mechanism of protein immobilization on silica involves electrostatic interaction with release of water at physiological pH, driven by the silanol/siloxide groups on the silica surface and positively charged residues on the R5 polypeptide [45, 46]. The efficiency of target R5 in enzyme immobilization on silica particles (Section 2.5, eq.1) was studied comparing the amount of protein immobilized for $R5_2$-mCherry-SOx-R5-6H with native SOx and mCherry. The immobilization of $R5_2$-mCherry-SOx-R5-6H on silica particles resulted in approximately 99% efficiency based on the inbuilt mCherry fluorescence $\lambda_{ex/em}$ = 587 / 607 nm of supernatant solution (Section 2.5, eq. 1) as can be seen in Fig. 2A and very selective to silica as a substrate (Fig. 2B). For solutions of purified $R5_2$-mCherry-SOx-R5-6H enzyme, loading on silica was proportional to the enzyme concentration in the incubation solution (Fig. 2C) with immobilisation efficiency independent of enzyme concentration (Fig. S1A).

In the absence of R5, only ~50% of SOx could be adsorbed non-specifically compared with ~99% R5-SOx. A higher level of adsorption was found with mCherry-6H (~80%) without the R5 (See Fig. 2A) but with loss of fluorescence (Fig. 2D). Red fluorescent proteins have been seen to have a susceptibility to loss of fluorescence, due to loss of secondary structure and denaturing on silica [47]. When mCherry-6H and R5-mCherry-6H are adsorbed onto a silica thin layer chromatography plate, Fig. 2D, mCherry-6H is adsorbed but the fluorescence is lost rapidly at room temperature for the native enzyme. In contrast, R5-mCherry-6H retained full fluorescence activity over 24 hrs, decaying only slowly and still retaining >60% of the original activity after one month (See Section 2.8). The R5-tagged mSOx is also resistant to leaching at normal physiological pH and even at alkaline pH. It could be partially released in acidic media (pH 4) after 30 min (around 40% of release). In addition, lysine (Lys) at high concentrations is able to competitively displace the R5 from the silica [48]. It can be seen in Fig. 2E that 85% of $R5_2$-mCherry-SOx-R5-6H was released using lysine at 1M pH 7.4, however these conditions are not representative of urine. In urine, loss of $R5_2$-mCherry-SOx-R5-6H from the silica was less than 4%.

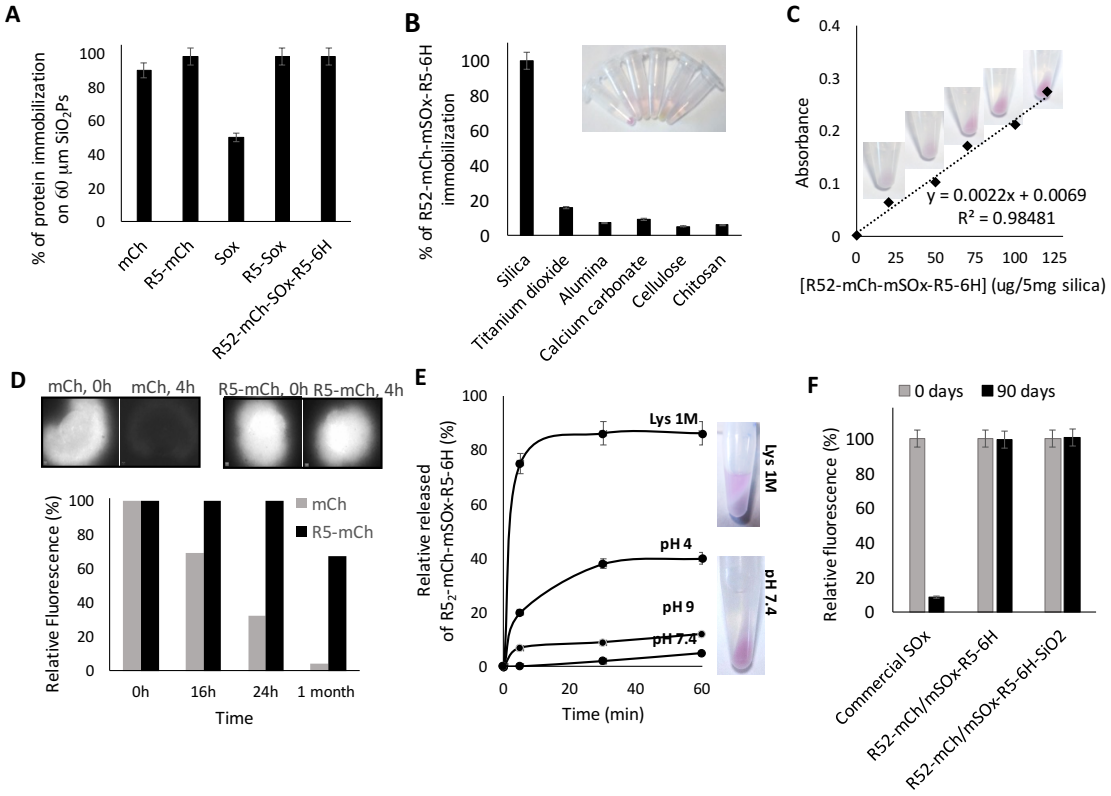

Fig. 2. A) Protein immobilisation on silica dependent on the presence or absence of the R5 silaffin polypeptide component for mCherry and SOx constructs (immobilisation from a solution of 0.4 mg/mL protein), B) selectivity of $R5_2$-mCherry-mSOx-R5-6H for silica compared with other substrates C) $R5_2$-mCherry-mSOx-R5-6H loading on silica depending of its concentration in the immobilisation incubation, D) Microscopy images of 1μL of mCherry-6H and R5-mCherry-6H (1 mg/mL) on thin-layer

chromatography (TLC) plate and the relative red channel fluorescence intensity (%) measured using an FFEI reader and analysed with ImageJ; E) $R5_2$-mCherry-mSOx-R5-6H leaching under strong conditions lysine 1M, compared with different pH; F) Stability of commercial wildtype SOx, $R5_2$-mCherry-mSOx-R5-6H in solution and the SiO_2- $R5_2$-mCherry-mSOx-R5-6H at room temperature for 90 days.

The silica used for the immobilization of the reagents can be obtained from natural resources such sand and from rice (Fig. S6) or purchased as narrow or broad range sized particles. Immobilisation efficiency (eq. 1) is influenced by silica particle size, and a decrease in immobilisation efficiency can be observed for larger particles. The best results were obtained for silica particles between 0.25 to 60 µm using concentrations of the protein between 0.2-0.4 mg/L (See Fig. S1B). This is also consistent with other work using the Car9 silica affinity peptide [49]. Silica of sand origin or silica particles of 60 µm were selected due to their easy precipitation with the protein, during the protein immobilization process, without need of centrifugation.

The effect of immobilisation on the enzyme activities was also highlighted for the wildtype SOx and $R5_2$-mCherry-mSOx-R5-6H, measured using the Trinder assay (See Section 2.6). Table 1 compares the specific activity for mSOx in solution and immobilised on silica using either the traditional chemical method of crosslinking with glutaraldehyde to an amino activated silica (see Section 2.4) or via the R5 affinity peptide. Although the solution of crude lysate containing $R5_2$-mCherry-mSOx-R5-6H had a relatively low specific activity of 1.33 U/mg, after selective immobilisation on to silica of the $R5_2$-mCherry-mSOx-R5-6H from the crude extract, this increases to 5U/mg, compared with 7.49 U/mg for the isolated purified protein. For comparison, the GA-linked SOx drops to 0.4 U/mg protein on silica, compared with 9.6 U/mg in solution.

Table 1. Enzymatic activities measured by Trinder's assay.

Enzyme	**mSOx**	**mSOx GA-**linked	**mSOx GA-**linked on silica	**$R5_2$-mCherry-mSOx-R5-6H**		**$R5_2$-mCherry-mSOx-R5-6H on silica** (apparent activity)	**HRP**	**HRP GA-**linked **on silica**
				Crude	**Pure**			
Activity (U/mg)	9.6±0.5	0.09±0.03	0.4±0.2	1.33±0.13	7.49±0.08	5 ± 0.3	323 ± 5	21 ± 1

The stability of the free and silica immobilised proteins showed clear differences when studied at room temperature over time (60 and 90 days) (Fig. 2). It is clear from this figure that the R5-mCherry fusions have produced a more robust protein with almost no loss of activity in 90 days, whereas the proteins without R5 lose more than 90% of their relative activity. The data for this

484 robust $R5_2$-mCherry-SOx-R5-6H provides a good basis for development of a sarcosine
485 biosensor.
486
487 3.2 Towards a sarcosine biosensor using SiO_2-$R5_2$-mCherry-mSOx-R5-6H
488 The enzyme-SiO_2 particle system allows different assay configurations to be considered. The
489 SiO_2-$R5_2$-mCherry-mSOx-R5-6H enzyme is very suitable for a packed column/bed format. Fig.
490 S5 shows the microfluidic system developed recently for an immunoassay biosensor [36],
491 repurposed here for an enzyme assay. In contrast as reported recently elsewhere [33], utilising
492 the principles of an hour glass and silica obtained from sand (Fig S6), Fig. S7 shows a thin film
493 hour-glass cell containing $R5_2$-mCherry-mSOx-R5-6H protein on silica. This was filled with
494 artificial urine and then inverted to cause the silica to fall through the sample and precipitate at
495 the base. Fig. S6 shows that by obtaining the change in the fluorescent intensity profile through
496 the cell for a given time interval (6 min was selected) a linear relationship with sarcosine
497 concentration is revealed. More conventionally Fig. 3A shows the calibration curve for
498 sarcosine with SiO_2-$R5_2$-mCherry-mSOx-R5-6H particles in artificial urine a simple eppendorf.
499 As can be seen from the plot, there is good linearity across the range of potential clinical interest
500 for identifying elevated sarcosine levels (>5μM) in patients with prostate cancer.
501
502 However. the assay can also be formulated to work as a spot test with very low volume samples
503 (eg 20μL, Fig. 3B) with a fluorescence output that can be read visually against an intensity
504 match card (see Fig. 3B) to provide a simple positive/negative result. A quantitative
505 measurement can also be obtained in this format with resolution adjusted for either high or low
506 sarcosine levels. For example, as can be seen in the plot in Fig. 3B, the reader intensity is set to
507 give a maximum at 3.5 μM (in the normal clinical range) so that measurement reaches signal
508 saturation at higher concentrations.
509
510 These preliminary results using artificial urine show promise for further construction of the
511 SiO_2-$R5_2$-mCherry-mSOx-R5-6H biosensor. However, this requires the coimmobilisation of the
512 HRP and Amplex Red.
513
514 3.3 Addition of HRP-SiO_2
515
516 The stoichiometric coproduct from the SOx catalysed oxidation of sarcosine is H_2O_2 which acts
517 as an indirect measurand of sarcosine, producing a colorimetric product catalysed by HRP (Fig.
518 1B). However, although horseradish peroxidase (HRP) is widely used in such combinations,
519 recombinant HRP is difficult to express and has a tendency to form inclusion bodies (IBs) in
520 E.coli with low yield [50] of active protein. Thus, commercially available peroxidases are

largely from plant extracts and are a mixture of acidic and basic isoenzymes. We have previously shown that some engineered type C R5-HRP isoenzymes, expressed in E.coli can be produced without IBs, but this isoenzyme has an activity of ~0.2U/mg compared with >300U/mg for the commercial mixed isoenzyme HRP of plant origin. In this instance, the wild type mixed acidic and basic HRP isoenzyme with high initial activity, is thus the preferred enzyme source for a first step in a peroxide linked sarcosine assay. This required a classical chemical immobilisation of HRP using GA coupling. This does not share the same benefits as the easy R5 protein modification, of being coupling-reagent free and single step, but provides a compatible stepwise model for taking the silica platform forward to explore whether sarcosine can be detected in urine.

In the HRP-silica containing samples, the FTIR (Fig. S2A) is dominated by a band at 1089 cm^{-1} which corresponds to Si-O-Si antisymmetric stretching vibrations. The presence of HRP is indicated by the characteristic absorptions at 1640 and 1540 cm^{-1}, attributed to -CONH- (amide I) and amide II, respectively (see insert in Fig. S2A). The HRP-SiO_2 particles are green and red autofluorescent (Fig. S2B) similar to observations in previous reports after glutaraldehyde crosslinking of serum albumins [51]. These authors attributed this observation to π–π^* transitions of the C=O bond and n–π^* transition of C=N bond as a result of the α,β unsaturated aldehyde.

The activity of HRP-SiO_2 particles (Section 2.6) compared with the free enzyme, showed the expected loss of activity following glutaraldehyde crosslinking (21±1 U/mg) (See Table 1) which is consistent with other reports of reduced activity following glutaraldehyde crosslinking [52] but nevertheless retains HRP activity at a higher level than the SOx, so that it should not limit the assay pathway. The SiO_2-$R5_2$-mCherry-SOx-R5-6H and HRP-SiO_2 can thus be combined for the determination of sarcosine and tested in artificial urine. As can be seen in Fig. 3C, the behaviour observed for the free HRP and HRP-SiO_2 in combination with SiO_2-$R5_2$-mCherry-SOx-R5-6H was broadly equivalent. This provides a good basis for a fully immobilised SiO_2-enzyme system.

3.4 Addition of Amplex UltraRed-SiO_2

The silica particulate format offers versatility for the assay, so to complete the presentation on silica, the Amplex Red group of dyes were encapsulated on the surface of silica particles within a sol-gel using TEOS in basic conditions. The dye-SiO_2 provided a vehicle for quick release and delivery of the reagent in contact with aqueous solution where the determination of sarcosine takes place. Under optimised conditions, with HRP-SiO_2 and dye-SiO_2 in sarcosine-spiked real

urine, the results showed that Amplex Ultrared (AR) has around 2-fold higher sensitivity than Amplex Red (Fig. 3D) in the range of 0-10 µM of sarcosine.

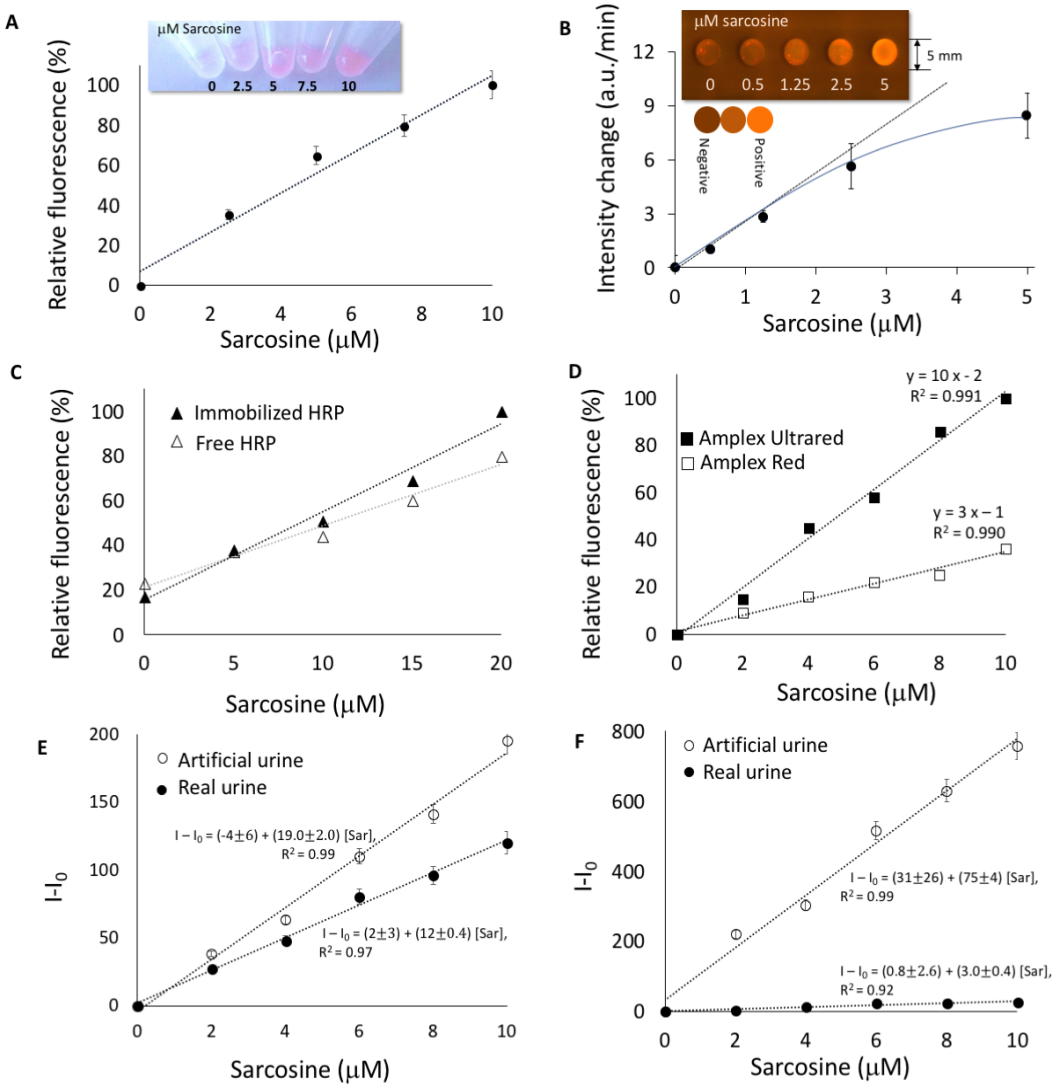

Fig. 3. Calibration curves for sarcosine determination in artificial urine using $R5_2$-mCherry-SOx-R5-6H-SiO_2 together with A), HRP and AR (100µL sample in eppendorfs); B) HRP and AR in a well-card showing a quantitative plot of fluorescence intensity. Visual fluorescent intensity image of well-card for 20µL samples shown with qualitative intensity image scale; C) AR and comparing the results with HRP and HRP-SiO_2; D) HRP-SiO_2 and comparing the sensitivity of Amplex Ultrared, AR (in black) against Amplex Red (in white) immobilized on silica E) HRP-SiO_2 and AR-SiO_2 compared with F) the commercial reagents SOx, HRP and AR in solution (note the different y axis scales). E and F tested in artificial urine and real urine from a control volunteer. Relative fluorescence measured at $\lambda_{ex/em}$ 530 / 582 experimental details in Section 2.7

573 3.5 Integrated sarcosine assay in urine

574 The pH of normal urine is within the range of 5.5 to 7 with an average of 6.2 [54] while urine
575 from cancer patients can be more acidic (pH 5). Amplex Ultrared has a broader useful pH range
576 from pH5 to 8 compared with Amplex Red from pH 6 to 7 (See Fig. S3B). This shows that the
577 pH should not affect the Amplex Ultrared for sarcosine determination in samples from either
578 negative controls or positive patients.

579

580 As mentioned earlier, the normal sarcosine test kit employing SOx suffers from interference for
581 measurements in urine, producing artificially low results (assay kits warn against use in urine,
582 but favour measurement in blood or serum, where sarcosine levels are not indicative for
583 prostatic carcinoma). This problem is also demonstrated here (Fig. 3F) using the commercially
584 available reagents in solution, where the urine sample, spiked with sarcosine produces a very
585 low fluorescence at 582nm, that is hardly sensitive to sarcosine concentration and only 4% of
586 the slope for artificial urine. This suggests that there is an inhibitor of the SOx or other matrix
587 effect for the reaction in the real urine. In contrast, the fluorescence intensity of the solutions at
588 582 nm for the silica-bound reagents showed a good linear relationship with sarcosine
589 concentration between 0 to 10 μM, with a slope in urine that is 63 % of that obtained in artificial
590 urine. It is evident that the silica-immobilised enzyme construct with SOx is less affected by the
591 urine. By calibration in spiked screened urine from a healthy patient, the system adjusts for this
592 background and provides excellent sensitivity in the required range, in contrast to the
593 commercial SOx kit.

594

595 It is known that the urinary amino acid alanine which is at higher concentration than sarcosine
596 in urine, is an interfering compound in sarcosine analysis by GC or LC coupled mass
597 spectrometry, due to their equivalent parent and fragment [53-55]. On the other hand, glycine,
598 which is a product of oxidative demethylation of sarcosine has also been shown to be a substrate
599 for SOx with low activity. Fig. S3A shows that alanine and glycine as well as some other
600 amino acids produced only a small fluorescent signal in the absence of sarcosine, as does uric
601 acid so that, alanine in particular, does not have the same significance as seen with coupled
602 mass spectrometry detection. Tyrosine and leucine appear to give a 'negative' signal, which
603 can be correlated with the direct interaction with the Amplex dye, reducing the background
604 fluorescence. Tyrosine dimerisation with H_2O_2 in the presence of HRP also forms a fluorescent
605 product[56], which can influence the final signal, although this effect is typically small at this
606 wavelength, since the emission is at a lower wavelength. These matrix effects are independent
607 of SOx concentration and can be accommodated in the background calibration with a combined
608 effect reducing the overall signal by ~20% on silica, but still retaining the required resolution
609 for sarcosine determination in clinical sample. In contrast, >80% reduction of the signal is

observed with the wildtype SOx in solution and the sarcosine test kits.

3.5 Application to urine from prostate cancer patients

The validity of the sarcosine sensor to discriminate between samples from healthy volunteers and prostate cancer patients was tested. The samples were processed without any pre-treatment and without dilution avoiding sample contamination and reducing the time of the assay. As mentioned before, the calibration slope obtained for artificial urine and urine was different, due to a matrix effect. In order to accommodate the matrix, the calibration curve was carried out in a real urine sample with a sarcosine content below the LOD. To evaluate the accuracy of the procedure, urine samples from cancer patients were also spiked with 5 and 10 µM of sarcosine. The recoveries obtained were near to 100% as can be seen in Fig. 4A suggesting that the calibration was robust. Three replicates were done in all the cases.

Five samples from healthy volunteers or controls were compared with 10 samples of prostate cancer patients using the proposed 'silica-enzyme' biosensor. Higher intensities were found for prostate cancer patients compared with the control (See Fig S4). The concentrations of sarcosine calculated were 0 to 2.2 µM and 6.8 to 10.6 µM from healthy and patient samples, respectively. In Fig. 4B, the sarcosine concentration of cancer patients is shown to be about 4 times higher than for healthy donors. As a first approach, the sensor demonstrated here appears to be able to discriminate between healthy and prostate cancer patients.

A

Spiked sample from a patient	Detected results	Recovery (%)
5	5.3 ± 0.4	106 ±8
10	10.6 ± 0.3	103± 4

B

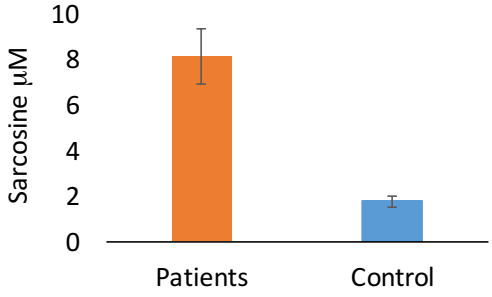

Fig. 4. A) The results obtained for a spiked sample from a patient with 5 and 10 µM of sarcosine (n=3). B) Comparison the mean concentrations of sarcosine estimated from urine samples of healthy volunteers (controls, n = 5) and PCa patients (patients, n = 10). For more experimental details see Section 2.7.

4. Conclusions

A novel fused protein $R5_2$-mCherry-Sarcosine oxidase-R5-6H was constructed which has the ability to self-immobilize on silica particles. The mCherry in the fused protein provided a useful visual reference throughout the production, isolation and immobilisation of the $R5_2$-mCherry-SOx-R5-6H. This protein showed higher stability than the commercial SOx, and the protein remained stable for at least 3 months. In addition, it can be calibrated in and remains sensitive in complex matrices such as urine, while the classical SOx assay suffers from interferences and loses sensitivity. $R5_2$-mCherry-SOx-R5-6H, HRP and Amplex UltraRed were all successfully immobilized on silica particles to develop a particle based solid state sensor for sarcosine determination in urine. No sample preparation or treatment was needed and the reaction took place in urine at room temperature in 10-20 min. The proposed sensor is sensitive in the required clinical range for sarcosine detection in urine. Furthermore, in this preliminary study, it was able to discriminate between urine from healthy and prostate cancer patients.

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