Organic & Biomolecular Chemistry

RSC Publishing

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c3ob40150b

Development of an *in situ* culture-free screening test for the rapid detection of *Staphylococcus aureus* within healthcare environments†

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This article reports the development of a novel fluorometric indicator which shows a rapid response when exposed to coagulase positive *Staphylococcus aureus* (SA) bacteria (including methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA) bacteria). The test is robust and will detect a wide variety of SA strains and there is no significant fluorescence response observed for other species of bacteria commonly found in clinical specimens, including other *Staphylococcus* bacteria. This research forms the basis of a prototype SA testing kit for the rapid detection of SA within hospital and healthcare environments as an economical prescreen or alternative to the current PCR based testing methodology. Rapid identification of SA carriers will allow hospital infection control teams to be pre-emptive and could significantly reduce the incidence of hospital acquired infections involving this organism.

Received 23rd January 2013,
Accepted 18th March 2013

DOI: 10.1039/c3ob40150b

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Introduction

O*Staphylococcus aureus* (MRSA and MSSA) infections are a significant problem within hospitals worldwide. In Europe alone, around 7% of all patients develop healthcare acquired infections and this is estimated to cost up to £11bn annually to diagnose and treat. Epidemiology suggests that up to 30% of the UK population are carriers of SA, currently UK hospitals only screen surgical patients.[1] It has been predicted that for an in-patient cohort of 70 000, cost savings of ~£600k could be achieved; along with the potential avoidance of 840 hospital acquired infections through the use of a routine pre-screen and treat strategy.[2] When this is considered in the context of the 26 million in-patient procedures performed within UK hospitals in 2010–11 (HESonline), this could lead to a potential national annual saving of £222 million and an annual reduction in the number of hospital acquired infections of 312 000 cases.

Current clinical detection methods for SA are limited by sensitivity. In all current detection methods, amplification of bacterial numbers *via* culturing or amplification of characteristic DNA *via* PCR are utilised for the detection of SA.[2] Only after augmentation of bacteria cell numbers can fluorometric molecular probes *e.g.* Boc-Val-Pro-Arg-amido-methyl-coumarin **2** or visual coagulase tests be used and only after PCR amplification can the hybrid DNA probes found within the BACLite™ assay be identified.[3,4] These amplification steps are time consuming (*ca.* 8 h for culture and 3 h for PCR) and require suitable instrumentation and skilled operators to obtain a result for a given sample.[3c,5] Couple this time delay to that associated with sampling and transport in a clinical setting and this can mean that a result for a sample may not be available until the next day for most cases.[6,7] These delays may make any infection more difficult and more expensive to treat, and increase the risk of the SA spreading to other patients before it has been identified/treated.[8] There are two key stages where SA detection can be sped up; these are the transport of a sample and the time required for culture or molecular detection prior to even a presumptive identification. Elimination of both of these limitations could be accomplished by the development of a simplified point-of-care assay, however to date no applications have been developed that are reliable, economical or robust enough to satisfy the requirements. It is clear that the development of an enhanced molecular probe with sufficient sensitivity to obviate the need for culturing would be a key step in shortening the time required for the detection of SA in a clinical environment.

Staphylocoagulase is an enzyme expressed externally by ~95% of all known SA bacteria. This enzyme normally

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†Electronic supplementary information (ESI) available: Full NMR of all compounds and HRMS of final compound along with additional biological data not included within paper. See DOI: 10.1039/c3ob40150b

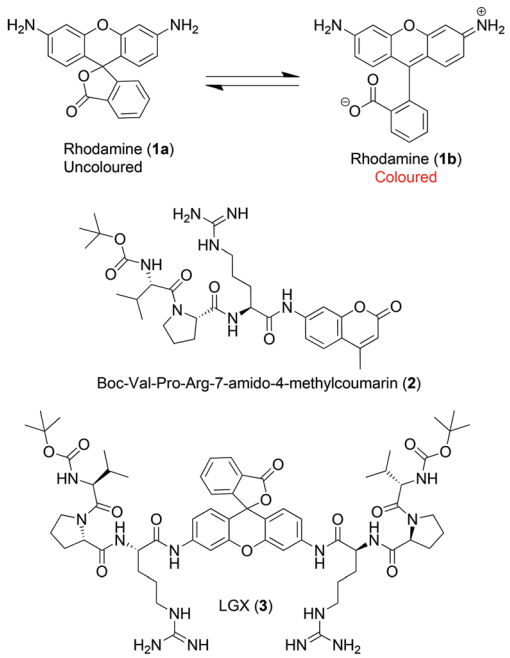


Fig. 1 Transition to coloured zwitterionic form of rhodamine **1**. The commercially available AMC probe **2** and our improved probe LGX **3**.

complexes with a second enzyme prothrombin to produce a staphylothrombin complex which naturally metabolises fibrinogen into fibrin by cleavage of a beta loop containing the amino acids X-Val-Pro-Arg-X.[9] The tripeptide Val-Pro-Arg has been shown to be an effective substrate mimic of fibrinogen, and the existing commercial fluorescent probe, Boc-Val-Pro-Arg-amido-methyl-coumarin (AMC) **2** utilises this enzymatic cleavage to release the fluorophore coumarin, which can be detected though changes in the fluorescence emission spectra.[3a,d] However, the sensitivity of the coumarin system is limited due to a low extinction co-efficient of *ca.* 4500 M^{-1} cm^{-1}, a relatively narrow bathochromic shift on loss of the tripeptide and a low overall molar fluorescence coefficient 4.15×10^9 RFU M^{-1} ($\lambda_{ex.}$ = 380 nm, $\lambda_{em.}$ = 460 nm), requiring a significant period of cell culture before bacterial cell count is significant enough for this test to be utilised.[10]

Rhodamine **1** is a commonly available dye that has been widely used in a number of biological applications, either as a cell stain or as a fluorescent tag.[11] It has a high extinction co-efficient of *ca.* 66 800 M^{-1} cm^{-1} and an overall molar fluorescence coefficient of 1.90×10^{11} RFU M^{-1} ($\lambda_{ex.}$ = 492 nm, $\lambda_{em.}$ = 523 nm).[12] Rhodamine also exists in equilibrium between two tautomers, a closed lactone tautomer **1a** which is colourless and an open zwitterionic tautomer **1b** which is strongly coloured (Fig. 1).[13]

Synthesis

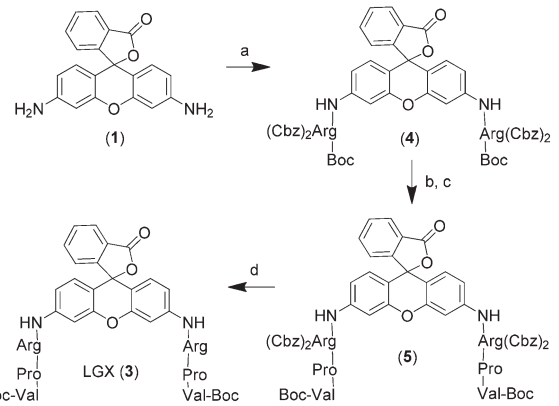
The principle of coupling peptides to rhodamine is not in itself, particularly new; many groups of enzymes *e.g.* serine proteases can be detected and quantified through coupling a peptide substrate to a chromophore/fluorophore,[12,14] however, there is no literature evidence for the successful coupling of the staphylocoagulase tripeptide substrate Val-Pro-Arg to rhodamine and we hypothesised that we could utilise this property to generate a more effective colour change indicator. The proposed rhodamine based probe **3** would function in a very similar manor to the commercially available AMC probe **2**, however, in the case of probe **2**, the difference in UV properties between the peptide bound and peptide unbound form are small, making this compound very difficult to observe at low concentrations, requiring lengthy cell culturing before it can be employed. Replacement of the coumarin with rhodamine would generate a colourless compound **3** that would become visibly coloured on exposure to MRSA/MSSA.

Due to the extinction co-efficient of rhodamine being many times that of coumarin, (68 800 *vs.* 4500 M^{-1} cm^{-1}), we predicted that detection could occur at a lower concentration of either dye or bacteria and/or obviate the need for the lengthy cell culture when compared to use of the coumarin dye system.

The synthetic route started with us first synthesising the tripeptide Boc-*N*-Val-Pro-Arg(NO_2)-OH through conventional liquid phase peptide coupling using EDCI and PFP ester methodology.[15] Before attempting to couple the tripeptide to the rhodamine, a model coupling of rhodamine to Boc-*N*-Arg-(NO_2)-OH using EDCI was carried out, however this was found to give a complex mixture of products with no obvious sign of the desired product after purification. A second model study was then carried out, coupling Boc-NH-Val-OH with rhodamine and after screening a range of coupling reagents, such as EDCI, EDCI/HOBT, EDCI/oxyma and COMU in a range of different solvents, times and temperatures, we obtained a moderate yield of the desired product using 4 equivalents of each of EDCI, Boc-*N*-Val-OH and NEt_3 in anhydrous DMF under microwave irradiation at 80 °C for 24 hours. Column chromatography of this mixture was hindered by the presence of both unreacted rhodamine and the mono-coupled Boc-Val-rhodamine, both of which exhibit two interconverting spots corresponding to the closed lactone and the open zwitterionic form, analogous to **1a** and **1b**. A 55% yield of purified (Boc-Val)$_2$-rhodamine was obtained after chromatography.

Application of these conditions for direct coupling rhodamine and the Boc-Val-Pro-Arg(NO_2)-OH tripeptide were attempted but it was found impossible to isolate an analytically pure sample of our target compound from this complex crude mixture. These coupling conditions were then replicated using a variety of different sidechain-protected arginines, such as NO_2, di-NO_2, Cbz, Pfp, di-Pfp and di-Cbz Boc-arginine. When Boc-*N*-Arg($CBz)_2$-OH was utilised, the number of side products was minimised, and it was possible to isolate an analytically pure sample of the (Boc-*N*-Arg($Cbz)_2)_2$-rhodamine **4** in an 18% yield (~90% pure samples could be obtained in a maximum of 42%).

With the arginine functionalised rhodamine **4** in hand, exposure to TFA in MeOH yielded **6** in 94%. The resulting glassy solid was then coupled with Boc-Val-Pro-OH using EDCI and oxyma in anhydrous DMF. After initial flash



Scheme 1 (a) HO–Arg(Cbz)$_2$NHBoc, EDCI, pyridine, DMF, 18%. (b) TFA, DCM, 94%. (c) HO-Pro-Val-NHBoc, COMU, NEt$_3$, DMF, 14%. (d) H_2, Pd/C, MeOH, DMF, 77%.

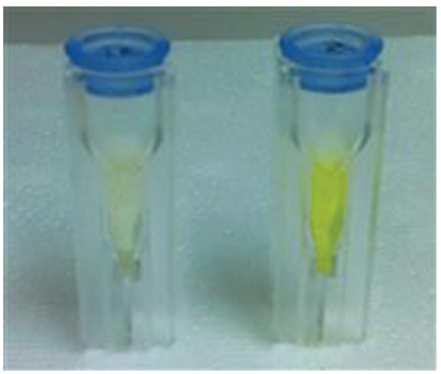


Fig. 2 Left: compound **3** in PBS buffer at 100 μM. Right: compound **3** in PBS buffer at 100 μM after exposure to MRSA coated inoculation loop and left for 1 h at 20 °C.

chromatography, NMR analysis was not conclusive, so further purification *via* preparative HPLC resulted in an analytically pure sample of **5** in an overall yield of 14% (Scheme 1).

This product was then dissolved in a 1 : 1 mixture of anhydrous DMF–methanol over 5% Pd/C before exposure to hydrogen at atmospheric pressure and temperature for 48 hours to yield the target (Boc-Val-Pro-Arg)$_2$-rhodamine **3**, which, after workup, was pure by NMR, in a yield of 77%.

UV-Vis spectra for **3** correlated with that for other rhodamine amide probes with a ν_{max} at 240 nm and similar extinction coefficient. There was no observed absorbance for **3** within the region of interest (400–600 nm) and the ν_{max} and extinction coefficient of rhodamine has been shown to be constant between pH 6 and pH 9.[16,17]

Biological testing

Preliminary testing involved simply preparing a 100 μM solution of LGX (**3**) in 1 M phosphate buffered saline solution (PBS). An inoculation loop was then streaked though a confluent growth of MRSA on an agar plate and the cells were suspended in a cuvette containing our LGX solution. Fluorescence readings were then taken over time and showed a steady increase in absorbance at 525 nm over time. In addition to this, on removing the sample from the spectrophotometer, a visible colour change was observed, raising the potential for a visual colourimetric readout to be obtained in the future clinical point-of-care test, significantly simplifying the testing process, see Fig. 2.

Screening was then carried out involving 15 clinical isolates of MRSA (as detailed in ESI†). These were cultured on nutrient agar overnight at 37 °C. Discrete colonies of each isolate were tested for coagulase activity using a staphylase test kit (Oxoid), following standard procedures, and each MRSA isolate was shown to be coagulase positive. Following this, an inoculum of each respective sample was cultured overnight in nutrient broth under aerobic, shaking conditions at 37 °C. Each sample was washed in sterile 1 × phosphate buffered saline (PBS) and varying concentrations of bacterial suspension, ranging from 10^0 to 10^6 colony forming units per mL (CFU mL^{-1}) were generated by serial dilution in 1 × PBS.

Solutions of 50 μM and 100 μM LGX were prepared aseptically (containing human prothrombin as detailed in the ESI†). In order to detect the presence of staphylocoagulase and thus the efficacy of LGX, varying cell concentrations (10^0–10^6) CFU mL^{-1} (50 μM LGX) and 10^0–10^4 CFU mL^{-1} (100 μM LGX) were added to a microtitre plate (Nunclon 96 well plates) in a 1 : 1 ratio with either 50 μM or 100 μM of LGX solution. This provided a final LGX concentration of 25 μM or 50 μM, respectively. The aspiration is to develop a visual indicator as a point-of-care test, however in order to determine the full sensitivity of our system at this early stage, the enhanced sensitivity afforded by fluorometry *vs.* colourimetry was utilised. Hand held fluorometers could also be utilised at the point-of-care in order to generate an accurate read out. The relative fluorescence was then recorded every fifteen minutes over a six hour time period ($\lambda_{ex.}$ = 488 nm and $\lambda_{em.}$ = 525 nm). Positive controls used in each experiment were a control strain of MRSA (NCTC 12493), and the negative controls were 10 clinical isolates of *Escherichia coli* (*E. coli*), the mean of which served as a control. A 1 : 1 ratio of 100μM LGX solution and 1 × PBS was used, resulting in a final LGX concentration of 50μM which was used as a control and termed "LGX alone".

As can be observed in Fig. 3, the results of all concentrations of MRSA (mean values for 15 bacterial strains at a given concentration) are plotted against the mean value of the negative control *E. coli* strains, at a concentration of 10^2 CFU mL^{-1} for both LGX concentrations (for demonstrative purposes). The mean value for LGX solution alone was used as a principal control for all SA strains, and a significantly greater increase in fluorescence intensity in MRSA samples was observed across the 6 hour time period when compared to the controls. Taking a lower threshold limit of 20 000 relative fluorescence units (RFU) as a positive result, it can be seen that at a 50 μM concentration of LGX, and at all bacterial concentrations of MRSA, there is a clear positive result (please see ESI† for 25 μM data). It is also noteworthy that there is an observed 'instantaneous' positive reaction, and this can be explained by the slight lag

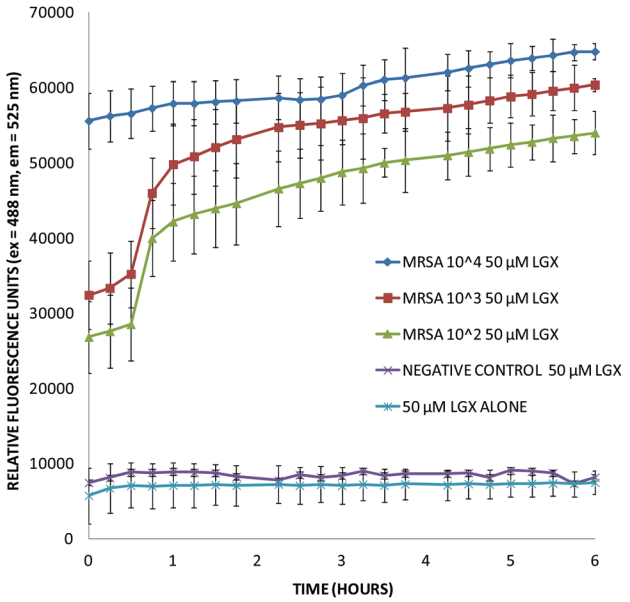


Fig. 3 The efficacy of 50 μM LGX in detecting MRSA concentrations as low as 10^2 CFU mL^{-1}. Average of fifteen bacterial strains (n = 3). Error bars represent standard deviation.

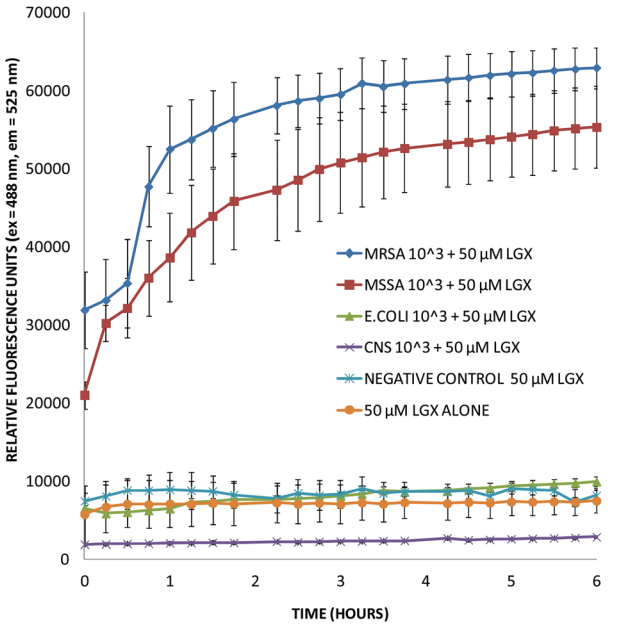


Fig. 4 The efficacy of 50 μM LGX in demonstrating both selectivity and sensitivity in detecting coagulase positive MRSA and MSSA, compared to coagulase negative *S. epidermidis and E. coli* at concentrations of 10^3 CFU mL^{-1}. Average of ten to fifteen bacterial strains (n = 3). Error bars represent standard deviation.

time between mixing, placing the sample in the fluorometer and the first reading, demonstrating the impressive rate of the initial reaction. This graph also highlights the stability of LGX–prothrombin mixture over the experimental time, which shows no obvious increase in fluorescence in the absence of bacteria, yet the presence of a staphylocoagulase containing microorganism rapidly stimulates a fluorescent response. The speed of the reaction is ascribed to the enzyme complex formed between staphylocoagulase and prothrombin, which is the active peptidase which cleaves the substrate mimic, at a rate relative to the bacterial concentration for each specific strain, demonstrating that the compound can rapidly detect SA concentrations of bacteria as low as 10^2 CFU mL^{-1}.

In order to determine whether this is a species-specific phenomenon and indeed whether the compound exhibits selectivity between bacterial strains (as detailed in the ESI†), 15 clinical isolates of each of MRSA, MSSA and *E. coli* and 10 strains of coagulase negative staphylococcal (CNS) species (*S. epidermidis*, *S. warneri*, *M. luteus* and *S. hominus*), were cultured on nutrient agar overnight at 37 °C. A sample of each type of bacteria was again maintained and tested for coagulase using a staphylase test kit (Oxoid), and each MRSA and MSSA was shown to be coagulase positive, and each *E. coli* and *S. epidermidis* was shown to be coagulase negative. Samples of 10^3 CFU mL^{-1} concentrations of each respective bacterial species were assayed under the same parameters as previously described.

As can be observed in Fig. 4, mean values for 15 bacterial strains at concentrations of 10^3 CFU mL^{-1} of bacteria amply demonstrate the efficacy of LGX as both a selective and sensitive means of determining the presence of coagulase positive bacteria. As previously described, all bacterial strains were assessed for coagulase activity, and the coagulase status of each bacterium juxtaposed with the results demonstrated in this experiment (staphylase test data available on request). There is a significant increase in fluorescence for coagulase positive MRSA and MSSA when compared to coagulase negative *E. coli* and *S. epidermidis* (please see ESI† for 25 μM data). It is important to note that this method of detection does not require the overnight culturing on agar which is necessary in performing the staphylase test, thus our method of determining the presence of SA, may be considered to be a culture independent method of bacterial detection, and is superior in terms of speed of results.

To further determine the efficacy of LGX it was necessary to compare it to the existing AMC probe **2**. A 500 μM or 100 μM of **2** (coumarin) solution was prepared with 1 × PBS as described for compound **3** (method adapted from Ford *et al.*, 1999),[18] and the complete solution was assayed as before with cell concentrations of bacteria (10^6, 10^5, 10^4, 10^3, 10^2 and 10^0 CFU mL^{-1}) in a microtitre plate. The coumarin probe **2** has previously been shown to be effective in the detection of MRSA at higher bacterial concentrations (~10^8 CFU mL^{-1}),[3d] and when mixed with other bacteria.[3e] Staphylocoagulase activity was determined by fluorescence spectrophotometry using literature precedent (λ_{ex} = 355 nm, $\lambda_{em.}$ = 460 nm) at 15 minute intervals over a 6 hour time period (n = 3 samples were assayed in each case).[3d] Strains of MRSA and MSSA were assayed for fluorescence intensity using 100 μM and 500 μM coumarin. This was compared to screens using 50 μM and 25 μM of LGX on identical bacterial samples.

Fig. 5 (for demonstrative purposes showing only concentrations of 10^3 CFU mL^{-1} and concentrations of 50 μM LGX and 500 μM coumarin – see ESI† for 25 μM LGX data) illustrates that the fluorescence produced by LGX, when compared to the coumarin system, is significantly greater, to the extent

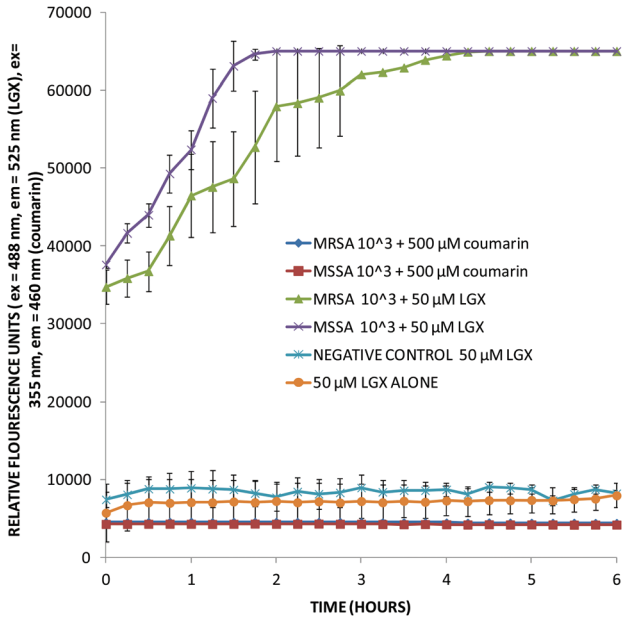


Fig. 5 MRSA and MSSA detection using LGX, compared to 500 µM coumarin, using bacterial concentrations of 10^3 CFU mL^{-1}. Average of ten bacterial strains (n = 3). Error bars represent standard deviation.

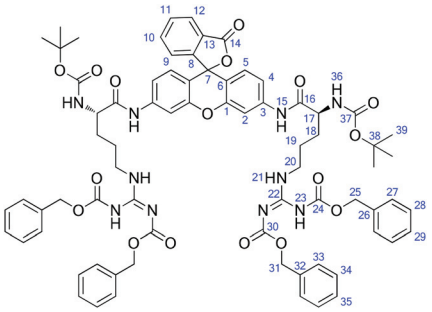
where one may consider the results incomparable. Even at 500 µM of coumarin, fluorescence fails to reach the lower threshold limit (20 000 RFU) for a positive result according to our LGX test. When comparing the efficacy of the LGX compound with the studies performed by Holliday *et al.*,[3d] whereby a bacterial cell concentration of 10^8 CFU mL^{-1} was necessary to produce a positive result with the coumarin system, our LGX system has proved to be far superior in terms of speed, selectivity and sensitivity, with it able to detect cell concentrations of >10^2 CFU mL^{-1} within 30 minutes.

Conclusions

We have developed a novel, selective fluorogenic assay, which obviates the need for time consuming culturing or genetic amplification, currently core to most hospital SA tests. Our test has proved itself to be rapid, presently giving a preliminary positive/negative result within 30 minutes for bacterial concentrations commonly found on skin (>10^2 CFU mL^{-1}). The testing methodology described within this paper is currently undergoing further biological screening within our labs and work is ongoing towards the development of a rapid point-of-care test kit.[19] This test kit would allow routine SA testing to become simplified, freeing up hospital microbiology from large scale SA screening and providing rapid preliminary results. This would allow hospital infection control procedures to become preemptive rather that responsive, reducing the costs, both monetary and impact upon infection, associated with treating hospital acquired SA infections. We are currently conducting research to further increase the sensitivity of this detection technique, alongside application towards a point-of-care diagnostic kit for use as an economical routine screening tool.

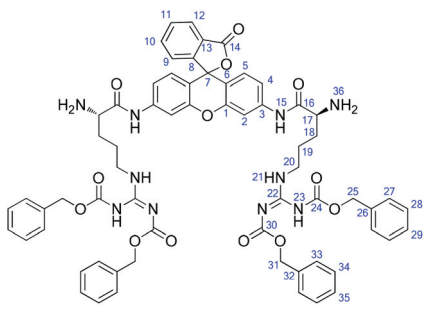
Experimental section

Rhodamine-(Arg-(CBz)$_2$-NHBoc)$_2$ (4)



To a solution of HO–Arg(Cbz)$_2$NHBoc (4.61 mmol, 2.5 g, 6 eq.), EDCI (4.61 mmol, 883 mg, 6 eq.), oxyma (4.60 mmol, 654 mg, 6 eq.) in anhydrous DMF (5 mL) was added freshly distilled anhydrous pyridine (5 mL) followed by rhodamine 110 (0.763 mmol, 280 mg, 1 eq.) and the solution was stirred at room temperature under a nitrogen atmosphere for 10 days. The reaction was then diluted with EtOAc (20 mL), washed with saturated aqueous $NaHCO_3$ (2 × 10 mL), 1 M aqueous LiCl (2 × 10 mL) and 10% aqueous $CuSO_4$ (3 × 10 mL), dried over $MgSO_4$ and concentrated to give a green oily solid. This crude product was then purified by column chromatography over silica gel, eluting with 25% EtOAc in $CHCl_3$ to yield the product as a white amorphous solid (0.14 mmol, 200 mg, 18%): δ_H (400 MHz, $CDCl_3$), 9.45 (2H, s, broad, NH-23), 9.29 (2H, s, broad, NH-21), 9.01 (2H, s, broad, NH-15), 8.02 (1H, dd, $^3J_{HH}$ = 6.02 Hz, $^4J_{HH}$ = 2.01 Hz, CH-12), 7.56–7.69 (2H, m, CH-10 and CH-11), 7.43, (2H, s, CH-2), 7.38–7.20 (20H, m, CH-27, CH-28, CH-29, CH-33, CH-34 and CH-35), 7.05 (1H, dd, $^3J_{HH}$ = 6.02 Hz, $^4J_{HH}$ = 2.01 Hz, CH-9), 6.77 (2H, d, $^3J_{HH}$ = 6.02 Hz, CH-4), 6.69 (2H, dd, $^3J_{HH}$ = 6.02 Hz, CH-5), 5.70, (2H, s, broad, NH-36), 5.30–5.02 (8H, m, CH_2-25 and CH_2-31), 4.39, (2H, m, CH-17) 4.07–3.87 (4H, m, CH_2-20), 1.86–1.62 (8H, m, CH_2-18 and CH_2-19), 1.41 (18H, s, CH_3-39); δ_C (100 MHz) 171.1, 171.0, 169.5, 163.6, 160.8, 156.2, 155.7, 153.1, 139.6, 136.4, 135.1, 134.4, 134.4, 129.7, 128.9, 128.8, 128.5, 128.4, 128.3, 128.0, 128.0, 126.2, 124.9, 124.1, 115.4, 114.2, 107.9, 82.4, 80.4, 69.1, 67.2, 54.8, 44.0, 28.5, 28.3, 25.0.

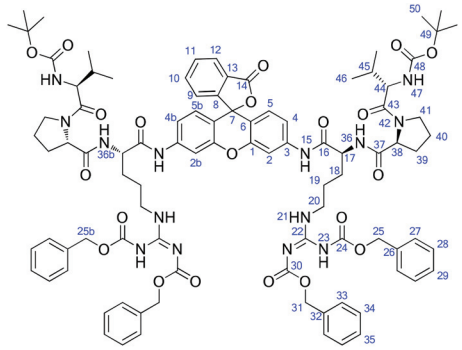
Rhodamine-(Arg-(CBz)$_2$-NH$_2$)$_2$ (6)



To a solution of Rho-(Arg-(Cbz)$_2$-NHBoc)$_2$ (0.14 mmol, 200 mg, 1 eq.) in DCM (2 mL) was added TFA (0.3 mL) and the reaction was stirred under nitrogen atmosphere for 6 hours. The reaction was then quenched with 1 M aqueous NaOH (1 mL) and extracted. The organic layer was then washed with saturated aqueous NaCl (3 × 1 mL), dried over $MgSO_4$ and concentrated to

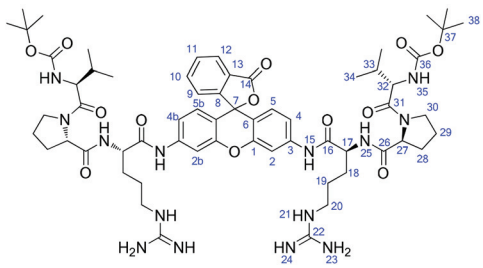
yield product as an amorphous solid which used immediately (0.13 mmol, 156 mg, 94%).

Rhodamine-(Arg-(Cbz)$_2$-Pro-Val-NHBoc)$_2$ (5)



A solution of Rho-(Arg-(Cbz)$_2$-NH$_2$)$_2$ (0.132 mmol, 156 mg, 1 eq.), HO-Pro-Val-NHBoc (0.264 mmol, 92 mg, 2 eq.) and NEt_3 (0.396 mmol, 40 mg, 55 μL, 3 eq.) in anhydrous DMF (1 mL) was cooled to 0 °C before the addition of COMU (0.264 mmol, 114 mg, 2 eq.). The reaction was stirred under a nitrogen atmosphere for 1 hour at 0 °C before warming to room temperature over a further 3 hours. The reaction was then diluted with EtOAc (2 mL), washed with saturated aqueous $NaHCO_3$ (2 × 1 mL), 1 M aqueous LiCl (3 × 1 mL) then dried over $NaSO_4$ and concentrated to give crude product. Product was purified by preparative HPLC to yield the pure product as a colourless glassy solid (0.0182 mmol, 32 mg, 14%): δ_H (600 MHz; $CDCl_3$) 9.42 (2H, m, NH-23), 8.83, (2H, s, NH-21), 8.05 (1H, d, $^3J_{HH}$ = 7.6, CH-12), 7.77 (1H, s, CH-2b), 7.70–7.61 (3H, m, CH-2, CH-10 and CH-11), 7.57 (1H, s, NH-36), 7.51 (1H, s, NH-36b), 7.41–7.36 (10H, m, CH-27, CH-28 and CH-29), 7.34–7.30 (4H, m, CH-33), 7.26–7.20 (6H, m, CH-34 and CH-35), 7.10 (1H, d, $^3J_{HH}$ = 6.4, CH-9), 7.09 (1H, d, $^3J_{HH}$ = 8.4, CH-4), 7.03 (1H, d, $^3J_{HH}$ = 8.4, CH-4b), 6.69 (1H, d, $^3J_{HH}$ = 8.4, CH-5), 6.68 (1H, d, $^3J_{HH}$ = 8.4, CH-5b), 5.27 (4H, s, CH_2-31), 5.20 (2H, d, $^3J_{HH}$ = 12.0, CH_2-25), 5.16 (2H, d, $^3J_{HH}$ = 8.6, NH-47), 5.06 (2H, d, $^3J_{HH}$ = 12.0, CH_2-25b), 4.53 (2H, m, CH-17), 4.34 (2H, m, CH-38), 4.25 (2H, m, CH-44), 4.07 (4H, m, CH_2-20), 3.73 (2H, m, C*H*H-41), 3.58 (2H, m, CH*H*-41), 2.07–1.66 (18H, m, CH_2-18, CH_2-19, CH_2-39, CH_2-40, CH-45), 1.47 (18H, s, CH_3-50), 0.97–0.84 (12H, m, CH_3-46); δ_C (125 MHz; $CDCl_3$) 172.0, 170.0, 163.5, 161.1, 155.9, 151.6, 140.0, 136.2, 135.0, 134.5, 129.7, 128.9, 128.9, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 126.3, 125.1, 123.9, 114.2, 108.2, 82.6, 79.7, 69.2, 67.2, 60.5, 60.3, 57.3, 57.2, 54.0, 47.7, 44.0, 31.3, 29.7, 28.4, 25.2, 25.0, 19.4, 17.5, 17.5, 14.2.

Rhodamine-(Arg-Pro-Val-NHBoc)$_2$ (3)



To a solution of Rho-(Arg-(Cbz)$_2$-Pro-Val-NHBoc)$_2$ (0.0182 mmol, 32 mg, 1 eq.) in anhydrous DMF (0.5 mL) and anhydrous MeOH (0.5 mL) was added 5% Pd/C (1 mg) and the mixture was stirred under a hydrogen atmosphere for 48 hours. The reaction mixture was then concentrated, dissolved in methanol and filtered through celite. Concentration yielded the product as an amorphous white solid (0.0140 mmol, 22 mg, 77%): δ_H (600 MHz; CD_3OD) 10.11 (2H, d, $^3J_{HH}$ = 7.7, NH-24), 8.48 (2H, d, $^3J_{HH}$ = 7.0, NH-25), 8.17 (2H, s, NH-15), 8.05 (1H, d, $^3J_{HH}$ = 7.6, CH-12), 7.89 (1H, s, CH-2b), 7.82 (1H, s, CH-2), 7.79 (1H, dd, $^3J_{HH}$ = 8.5, $^4J_{HH}$ = 6.9, CH-11) 7.73 (1H, dd, $^3J_{HH}$ = 8.5, $^4J_{HH}$ = 6.9, CH-10), 7.41 (2H, s, NH-21), 7.22 (1H, d, $^3J_{HH}$ = 7.8, CH-4), 7.21 (1H, d, $^3J_{HH}$ = 7.8, CH-9), 7.19 (1H, d, $^3J_{HH}$ = 7.8, CH-4b), 6.75 (2H, d, $^3J_{HH}$ = 7.8, CH-5), 6.75 (2H, d, $^3J_{HH}$ = 7.8, CH-5b), 6.65 (2H, $^3J_{HH}$ = 7.8, NH-35), 4.52–4.96 (4H, m, CH-17 and CH-27), 4.20 (2H, m, CH-32), 3.93 (2H, m, CH*H*-30), 3.70 (2H, m, C*H*H-30) 3.24 (4H, m, CH_2-20), 2.28 (4H, m, CH_2-18), 2.12 (2H, m, CH*H*-28), 2.04–1.96 (6H, m, C*H*H-28, C*H*H-29 and CH-33), 1.87 (2H, m, CH*H*-29) 1.45 (18H, s, CH_3-38) 1.01 (6H, m, CH_3-34) 0.96 (6H, m, CH_3-34); δ_C (125 MHz; CD_3OD) 173.2, 172.2, 171.0, 169.8, 163.8, 157.4, 156.7, 151.5, 140.7, 135.4, 130.0, 128.0, 126.2, 124.6, 123.7, 115.6, 114.1, 107.4, 82.7, 79.2, 73.3, 60.4, 58.0, 53.7, 48.6, 48.3, 44.1, 40.7, 35.6, 30.2, 29.4, 29.2, 28.9, 27.3, 24.9, 24.7, 22.3, 18.4, 17.2. (3 additional peaks for C-2b, C-4b and C-5b); *m*/*z* (CI) 618.3317 [M + 2H]; HRMS: Found 618.3322 (*z* = 2) [M + 2H], $C_{62}H_{86}N_{14}O_{13}$ [M] requires 1235.4322 (*z* = 1), 617.7161 (*z* = 2).

Acknowledgements

The authors would like to thank Kingston University, Enterprise and PARC for the funding to support this project, as well as the EPSRC mass spectrometry service, Swansea, UK, for providing high resolution mass spectra.

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