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Application of fluorescent substrates to the *in situ* detection of prostate specific antigen

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ABSTRACT

The forensic identification of body fluids frequently presents an important source of genetic material and investigative interpretation. However, presumptive testing techniques presently employed in the discrimination of biological fluids are subject to criticism for poor specificity, lack of fluid localisation ability and detrimental effects on DNA recovery rates. The recognition of fluid-specific biomarkers by fluorogenic substrates may provide a novel resolution to these issues but research has yet to establish any pertinent *in situ* fluid detection applicability. This study therefore utilises a fluorogenic substrate (Mu-HSSKLQ-AFC) specific to the seminal protein prostate specific antigen in an effort to detect human semen deposited on a number of surfaces typical to criminal investigation. The ability of fluorescent fluorogenic substrates to simultaneously identify and visualise biological fluids *in situ* is demonstrated for the first time, whilst the production of complete STR profiles from fluid sources is also confirmed to be completely unaffected by substrate application.

1. Introduction

Locating and identifying body fluids such as semen, blood and saliva can often aid the progression of criminal investigation by providing intelligence on the nature and circumstance of an offence and may additionally associate or exonerate a suspect through the isolation of genetic material.

A number of 'presumptive' screening assays exist to rapidly exclude or indicate fluid presence, employing simple biochemical processes in order to generate colorimetric changes within a given substrate. Those indicating the presence of blood rely on the oxidation of haem to catalyse substrate-specific reactions [1–3], whilst intra-fluidic enzyme activity provides the basis for the testing of semen and saliva [4,5]. However, previous validation studies have established limitations in the usefulness and evidential strength of these assays. With the exception of the chemiluminescence phenomenon exploited in the detection of blood by Luminol, presumptive tests cannot be used to localise fluid depositions, thereby necessitating time-consuming visual searches prior to analysis. Furthermore, the molecular targets examined by these tests are not fluid-specific, often leading to false positives between different fluid types and other non-fluid substances [6–8]. Detrimental effects on the recovery of DNA from fluid depositions have also been demonstrated after some presumptive test applications [9,10].

Currently the most widely used presumptive test for semen identification is the Brentamine assay for the detection of acid phosphatase, an enzyme secreted into semen by the prostate gland [4]. However, the requirement of specialist knowledge and equipment often makes this test problematic. Results are subject to a high level of expert interpretation [11–13], whilst Brentamine toxicity also necessitates use of a fume hood.

Recent improvements in fluid assay specificity have utilised immunological testing strips for the detection of fluid-endogenous protein biomarkers [14–16]. However, these testing processes do not allow for the retention of fluids following application, potentially sacrificing a valuable source of material for genetic profiling [17].

Our research group has made initial efforts in the design of novel body fluid analysis techniques, developing a fluorescent biosensor complex specific to Glycophorin A, an erythrocyte membrane protein used in the identification of human blood [18]. However, whilst demonstrating effective glycophorin detection via decreases in fluorescence intensity, the 'turn-off' nature of signalling restricts the use of this sensor in visualising discrete fluid deposits *in situ*. A 'turn-on' fluorescence based assay is therefore preferable for simultaneous identification and localisation purposes.

The proteolytic digestion of peptide substrates to release fluorescent by-products within the same molecular unit may be considered an attractive signalling mechanism for *in situ* fluid detection. High specificities make enzyme recognition elements ideal candidates for fluid analysis, whilst 'turn-on' increases in fluorescence

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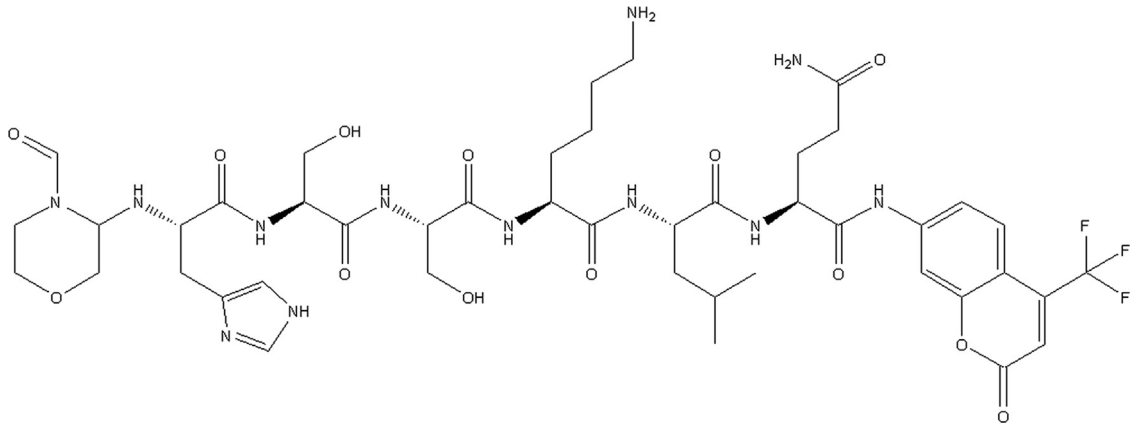

Fig. 1. PSA fluorogenic substrate – a hexapeptide consisting of amino acid sequence HSSKLQ, terminally labelled with a fluorescent coumarin derivative. Specific digestion of the peptide by PSA yields highly fluorescent 7-amino-4-trifluoromethyllcoumarin.

intensity upon target interaction may allow the visualisation of *in situ* fluid depositions. Furthermore, unlike irreversible antibody-based reactions, enzyme targets may interact with multiple substrate molecules to amplify signal production. Simultaneous detection of multiple fluid enzymes may also potentially be achieved by exploiting fluorophores of differing wavelengths in a single multiplex assay.

A central appeal of fluorogenic substrates as an alternative to current presumptive assays is that DNA-degrading oxidative processes, such as those exploited by Luminol and Leucomalachite green, are not required to generate a positive response [6]. With research yet to explore the effect of substrates on genetic material, investigation into the possible interference of reagents with DNA amplification, quantitation or profiling may be considered pertinent.

This study therefore explores the use of fluorogenic peptide substrates specific to prostate specific antigen (PSA) for the simultaneous visualisation and identification of human seminal fluid. PSA is a semen-endogenous protein responsible for proteolysis of gel-forming Semenogelin 1 and 2 [19]. The unique expression level of PSA within seminal fluid, often produced in milligram levels per millilitre [19], has established its wide acceptance as a forensic biomarker for semen identification.

Denmeade et al. [20] produced 12 peptide substrates for monitoring PSA activity based on amino acid sequences directly adjacent to mapped PSA cleavage sites of Semenogelin 1 and 2. These substrates utilise 7-amino-4-methylcoumarin fluorophores, which after amide bond conjugation to peptides undergo excitation and emission wavelength shifts, restricting fluorescence output. Subsequent separation of the fluorophore from the peptide by serine protease hydrolysis occurs in the presence of PSA and restores fluorescence.

The particular substrate MU-HSSKLQ-AFC (Fig. 1) displayed the highest specificity for PSA, arising from its resistance to similar proteolytic enzymes found within body fluids. Whilst this substrate has found routine use in the recognition of prostate cancer markers, it has yet to be applied towards the detection of human semen.

The fluorescence response of substrate MU-HSSKLQ-AFC to dilutions of semen within solution, as well as to whole semen extracted from *in situ* swabs, was measured via spectrofluorometry to determine the ability of the fluorogenic substrate to detect free PSA within seminal fluid. Further *in situ* detection ability was examined, testing substrate performance against semen deposits on glass slides and a number of surfaces typically encountered within forensic casework. Assay reagent was also applied to depositions of blood, saliva and urine to confirm substrate specificity. MU-HSSKLQ-AFC was lastly applied to semen samples for subsequent SGM plus profiling to assess reagent effect on each stage of the profiling process.

2. Materials and methods

2.1. Reagents

2.1.1. Fluorogenic PSA substrate

Lyophilised Prostate Specific Antigen Fluorogenic Substrate (Mu-HSSKLQ-AFC) was purchased from EMD Millipore (Massachusetts, USA) and dissolved in 109.5 μl DMSO to make an 8 mM stock solution before dilution in PBS to a working concentration of 400 μM.

2.1.2. Body fluid collection and storage

Blood, semen, saliva and urine samples were taken after informed consent. Blood samples were drawn by venipuncture and stored in a BD Vacutainer® Plus tube (Oxford, UK) containing 3.2% sodium citrate coagulation preservative. All tissue samples were stored at 4 °C until analysis.

2.2. Instrumentation and procedures

2.2.1. Spectrofluorometry

Fluorescence measurements were conducted on a BioTek Synergy HT spectrophotometer (Vermont, USA). Dilution curves were constructed through the addition of 100 μl of diluted semen (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) in a 96-well microplate to 100 μl of 400 μM PSA fluorogenic substrate and measured with appropriate blank (200 μl PBS) and negative controls (100 μl PBS, 100 μl assay reagent). Swabs taken from *in situ* semen depositions were extracted in 100 μl of PBS and added to 100 μl of working concentration substrate. All fluorescence emissions were recorded at room temperature in duplicate using Ex400/Em528 ± 20 nm wavelengths (for the measurement of emissions at 508 nm) immediately after mixing.

2.2.2. Slide microscopy

Fluorogenic PSA reagent was tested against seminal dilutions (1:25, 1:50, 1:100, 1:200, 1:500, 1:1000) deposited on glass slides as a demonstration of *in situ* substrate sensitivity. Semen volumes of 10 μl were applied to the centre of each slide before the direct 10 μl addition of substrate. Duplicates of each dilution were performed. Negative reagent-only controls were applied on the same slide as a measure of background reagent fluorescence, whilst blank controls consisting of semen-only applications were also used to monitor possible analyte auto-fluorescence. The simulation of dry depositions was achieved through the application of 10 μl of seminal fluid to glass slides, which were subsequently allowed to dry overnight. Reagent was then applied directly at the point of analysis.