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# Enabling fluorescent biosensors for the forensic identification of body fluids

Nunzianda Frascione,* James Gooch and Barbara Daniel

The search for body fluids often forms a crucial element of many forensic investigations. Confirming fluid presence at a scene can not only support or refute the circumstantial claims of a victim, suspect or witness, but may additionally provide a valuable source of DNA for further identification purposes. However, current biological fluid testing techniques are impaired by a number of well-characterised limitations; they often give false positives, cannot be used simultaneously, are sample destructive and lack the ability to visually locate fluid depositions. These disadvantages can negatively affect the outcome of a case through missed or misinterpreted evidence. Biosensors are devices able to transduce a biological recognition event into a measurable signal, resulting in real-time analyte detection. The use of innovative optical sensing technology may enable the highly specific and non-destructive detection of biological fluid depositions through interaction with several fluid-endogenous biomarkers. Despite considerable impact in a variety of analytical disciplines, biosensor application within forensic analyses may be considered extremely limited. This article aims to explore a number of prospective biosensing mechanisms and to outline the challenges associated with their adaptation towards detection of fluid-specific analytes.

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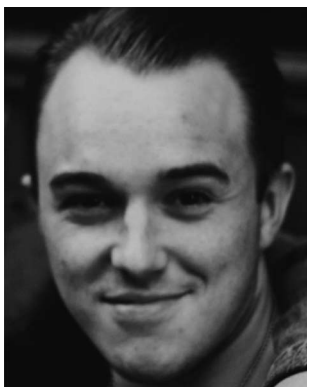
## Introduction

Determining the presence of biological fluid depositions may be considered paramount to many forensic investigations, with fluid detection and identification often elucidating the circumstance and severity of a criminal offence. Furthermore, successful fluid recovery has the propensity to yield a highly valuable source of genetic material, which may in turn associate an individual with a specific place, object or event.

However, unless immediately visible, ascertaining the location of a fluid deposit can be very challenging, with a meticulous visual examination by trained personnel presently being the most effective method of detection. It is consequently likely that

*Department of Forensic and Analytical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, UK SE1 9NH. E-mail: nunzianda.frascione@kcl.ac.uk; Fax: +44 (0)020 7848 4980; Tel: +44 (0)020 7848 4978*

*Nunzianda Frascione completed a five-year MSc in Pharmacy at "Federico II" University of Naples (Italy). In 2009 she was awarded a PhD in Molecular Biology. The work carried out during her doctoral studies embraced diverse research areas including biotechnology, nano- and materials science. In 2010 she joined Dr Daniel's group at the Department of Forensic Science and Drug monitoring (King's College London) where she became involved in forensic and anti-doping research. The development of new methods for the detection and identification of biological materials in forensic casework represents one of her main research focuses.*



*James Gooch obtained his BSc in 2011 from Bournemouth University before completing his MRes in Forensic Science at King's College London. He is currently continuing at KCL, working towards gaining his PhD through a research project on novel body fluid identification strategies.*

small or transparent fluid traces, particularly those deposited on dark backgrounds may often go unobserved. Detection may be aided by exploiting the auto-fluorescent properties of some intra-fluidic molecules through excitation with specialised light sources, but is often limited by fluid and evidential surface type.

On the proviso that a potential fluid deposit is located, point-of-care testing is usually performed in an attempt to establish the fluid type. Successful identification may not only provide a source of investigative information, but also prevents a costly downstream genetic analysis on stains that are found not to be of a biological origin. However, these tests are penned as 'presumptive' in nature as they cannot conclusively confirm fluid identity and have been shown to produce both false positive and false negative results. The demand is therefore apparent for new fluid detection strategies that would allow for the simultaneous localization and accurate identification of biological fluid deposits.

Exploration into the innovative world of fluorescent 'biosensor' technology shows exciting potential for the development of novel body-fluid identification methods that may enable the non-destructive and real-time detection and localization of scene depositions based on interaction with highly specific intra-fluidic macromolecular targets.[1–3] Successful employment of these mechanisms is likely to lead to a significant reduction in the labour expense associated with current manual stain search and identification strategies.

The intention of this review is to therefore explore the relative weaknesses of current biological fluid detection methods and to discuss the adaptability of biomolecular assay mechanisms based on biosensing technology towards the detection and identification of body fluids.

Particular emphasis is given to those sensing mechanisms which, in a forensic scenario, would allow the simultaneous localisation and identification of body fluids through the detection of specific analytes (either in solution or deposited onto surfaces). For such reasons, only optical sensing mechanisms, in which a fluorescent signal is produced upon binding, are covered herein as the most suitable signalling method for such purposes.

*Barbara Daniel started her academic life in immunology but soon moved to forensic science and is now the head of the Forensic and Analytical Science Department at King's College London. She is particularly interested in the cross-over of ideas from mainstream science to forensic science. The restrictions and constraints imposed by working in a non-lab based, unsterile, multi-substrate, 'dry' environment is a challenge she thinks can be met by the use of biosensors. Her research group has had success working on the detection of biological material using these methods in recent years.*

## Present technology and recent advances

Blood, semen and saliva are the biological fluid samples most frequently encountered within criminal inquiries, whilst additional depositions such as urine and sweat also occasionally provide a beneficial source of investigative information. A number of preliminary identification techniques exist for each respective biological fluid, acting as a simple indicative or exclusive screening test prior to the subsequent laboratory-based confirmation required for evidential use. The variability of scene locations ensures that these fluids are likely to be deposited on a diverse array of surface materials. It is therefore vital that presumptive testing methodologies are sufficiently robust to permit correct identification regardless of deposition medium.

Presumptive testing techniques presently employed to indicate or exclude a potential source of biological fluid deposition generally rely upon the use of simple biochemical reactions. Products such as Brentamine Fast Blue acid phosphatase and Phadebas® amylase testing reagents exploit the hydrolytic properties of intra-fluidic enzymes in order generate a colorimetric change for the respective identification of semen and saliva.[4,5] Other assays including Luminol, Phenolphthalein Kastle-Meyer and Leucomalachite Green are used for the identification of blood and exploit the catalytic action of haem groups present within haemoglobin in order to stimulate a substrate-specific reaction.[6–8] However, currently employed techniques are generally limited by one or more of the following aspects:

- interference with DNA profiling and sample destruction;
- inability to be used in a multiplex reaction;
- inability to spatially locate body fluid depositions;
- lack of specificity.

Studies have shown that reagents presently employed in some identification techniques adversely affect the recovery rate of the genetic material by destroying the samples or by inhibiting downstream reactions.[9,10] Often these tests require a portion of the stain to be removed from the substrate; after testing, the sample can no longer be used for further analyses (*e.g.* DNA profiling). The destructive nature of these methods results in a loss of biological material that would be detrimental in those cases in which only limited amounts are available. In such circumstances, where minimizing the loss of genetic material is especially pertinent, suspected body fluids may even forgo a presumptive identification step and proceed directly to DNA analysis, prompting a potential loss of investigative information. Conversely, there are in fact times when knowing which type of body fluid provided the DNA is of vital importance as this information can have a probative value and can strengthen the significance of DNA profiling.

Another limitation of current techniques is that they cannot be used in conjunction in one multiplex reaction. There is no single methodology to simultaneously identify an unknown forensic sample against all possible body fluids. Each body fluid test must be performed separately, consuming time and evidence, as well as requiring multiple instruments and trained laboratory personnel.

The majority of current mechanisms also suffer from the inability to localize a biological fluid deposition whilst its position within a scene or upon an object is undetermined, thereby confining testing to a limited area on the basis of circumstantial assumptions and manual searching restraints. This has particular relevance to the detection of latent stains, which cannot be observed without enhanced visualization, or those that resemble other non-fluid substances. This limitation carries the risk of missed evidence and, as demonstrated by recent high profile cases, can compromise the outcome of an investigation.

Attempts to increase search efficiency have largely centred upon the use of alternate light sourcing, which utilizes the auto-fluorescent properties exhibited by some fluids upon wavelength-specific excitation. However, this fluorescence is often conditional, with demonstrated signal variability between fluid types and deposition surface materials.[11,12] With few exceptions, existing presumptive tests do not demonstrate high degrees of specificity for biological fluid depositions and have been proven to cross-react with an assortment of non-fluid substrates.[9,13,14] This is especially prevalent with regard to the reagent peroxidation mechanisms exploited in the detection of blood, which provide false positives for a range of oxidative products endogenous to the sampling environment.[15,16] Furthermore, without unique human-specific fluid component interactions, the simple nature of testing chemistry remains incapable in the differentiation of human fluids from their non-human analogues.[9,14,17] The need for specific and non-destructive testing that is able to simultaneously identify and locate body fluids is demonstrated by the growing research in the field. Several new techniques have been developed in recent years with the purpose of circumventing many of the issues associated with current preliminary testing.

Tests based on immunological interactions, such as the Rapid Stain Identification (RSID) tests for blood, semen and saliva and the ABAcard systems for the detection of P30 protein antigen within seminal fluid, have been manufactured and offer a higher degree of specificity through target-sensitive antibody binding.[18–20] Although solving specificity issues, stain swabbing or surface medium excision is still required for the extraction of the testing material. Therefore such assays cannot localize fluid deposits on an evidential surface or be used in a single multiplex test. Promising results have been obtained through the forensic application of RNA profiling; because each body fluid has a unique pattern of RNA, these differences can be exploited for the development of a body fluid identification method.[21,22] RNA markers utilised are highly sensitive and the possibility to be combined into a multiplex assay has already been demonstrated.[23] Challenges are posed by the well-documented instability of RNA and its tendency to degrade, this being particularly accentuated in forensic samples for which control over environmental conditions cannot be achieved. Based on similar scientific principles, DNA methylation analysis[24,25] has also been introduced to the field of body fluid identification. However, the application of this test is still in its infancy, hindered by methodological limitations as well as by specificity and sensitivity issues. Moreover, none of the aforementioned nucleic acid-based tests has the potential to be used as an *in situ* detection method, leaving the localization of stains a largely unresolved issue.

Focus on the use of Raman spectroscopy has achieved the non-destructive differentiation of several fluid types and their non-human equivalents by utilizing the inelastic scattering of monochromatic laser light upon interaction with molecular vibrations to identify intra-fluidic molecular components.[26,27] The recent development of portable Raman spectrometers may consequently provide relevance of this technique to the *in situ* detection of biological fluid depositions at crime scenes.[28] However, without full device automation it may be argued that successful differentiation will require specialist knowledge in the interpretation of mixed or contaminated output spectra.

Recent work has demonstrated the successful *in situ* detection and differentiation of erythrocytes and nucleated leukocytes within human blood *via* the interaction of blood-specific antigens with fluorescently labelled antibodies.[29] Although this method allowed detection at a single cell level, proving suitable in aspects of sensitivity and specificity, the non-homogenous nature of this assay necessitates a series of washes in order to remove the remaining unbound antibody fraction. There is therefore potential for these wash steps to negatively affect the recovery of DNA from a source of biological staining.

Further work sought to overcome this limitation through the utilization of iron-oxide nanoparticles conjugated directly to the antibody–fluorophore complex which allowed the unbound fraction to be removed magnetically.[30] Despite resolution of issues regarding specificity and DNA recovery, magnetic removal may be considered cumbersome, with an indication of true complete unbound removal remaining absent, potentially resulting in the overestimation of detectable analyte quantities. An ideal resolution to these issues would likely arise from the direct spray application of biological sensing units, able to generate an easily observable signal without the need for associated enhancements or isolation steps (Fig. 1).

## Biosensors

Emerging research into the exploitation of analytical 'biosensors' has previously demonstrated the delivery of real-time information regarding the presence and activity of specific molecules within complex sample matrices for many biological

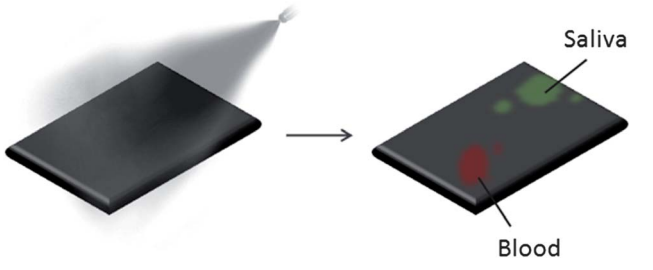



**Fig. 1** Ideal *in situ* application of biosensors to the detection of body fluids. Sensing molecules are dispersed across surfaces *via* aerosol, generating different fluorescent wavelengths upon multiple fluid target interaction.

and medicinal applications. Biosensing is usually achieved by use of a supramolecular complex comprising of two components; a sensitive biological detection element for the recognition of a testing antigen and a physiochemical transduction element that stimulates production of an observable output upon successful interaction.[31] Biosensing assays may be regarded as a single integrated system, with direct spatial contact between recognition and transduction elements. Potential for the application of biosensing technology towards the identification of biological fluids arises from a high molecular binding selectivity, combined with a non-destructive and non-invasive testing nature. As signal output transpires only upon an established biological interaction event, there is no prerequisite for the removal of any remaining un-transduced reagent prior to measurement. Detection based on sensing may therefore be considered entirely homogenous, as it does not require additional sample component processing, enhancement or visualization. This lack of sample extraction or isolation steps is consequently likely to mitigate the risk of DNA destruction during fluid identification.

Biosensor construction is facilitated by a large design flexibility, with a wide range of recognition moieties, signal outputs and intrinsic transduction mechanisms available to meet specific assay requirements. Components such as enzymes, antibodies and nucleic acid sequences may be utilized for biological recognition *via* simple conjugation chemistry, allowing the detection of almost any molecular target.[31] These components may overcome the low specificity associated with the simple chemical processes of current fluid detection techniques by selective interactions with proteins unique to body fluid residues. Such proteins are well characterised and, as previously mentioned, are already in routine employment in immunological fluid testing (Table 1).

Sensing output exists in many different formats, including electrochemical, thermal, magnetic or colorimetric signal detection. Identifying an array of fluid-specific analytes may require a low-cost general sensing strategy that employs simple instrumentation applicable to scene analysis. Such qualities are often afforded by fluorescent biosensing mechanisms, in which versatile and sensitive detection is provided in extremely rapid response times.[40]

Depending on the transduction mechanism, biological interaction can cause various fluorescence changes during sensing, either turning a signal on or off, or shifting its output wavelength. (Fig. 2). For fluid detection and identification purposes, turn-off reporting mechanisms may be considered unsuitable, as while potentially allowing confirmation within a known solution, a decrease in observable signal prevents the simultaneous localization of fluid depositions *in situ*.[41] Conversely, a turn turn-on signal, in which fluorescence is produced only after biosensor–target interaction, or a shift mechanism, in which the detection of the analyte causes a change in the emission wavelength of the biosensor, would both provide systems that allow deposit visualisation. Furthermore, the routine employment of instrumentation required for fluorescence detection, including excitation light sources and sensitive CCD cameras, ensures that that no additional specialised knowledge is required for biosensor application.

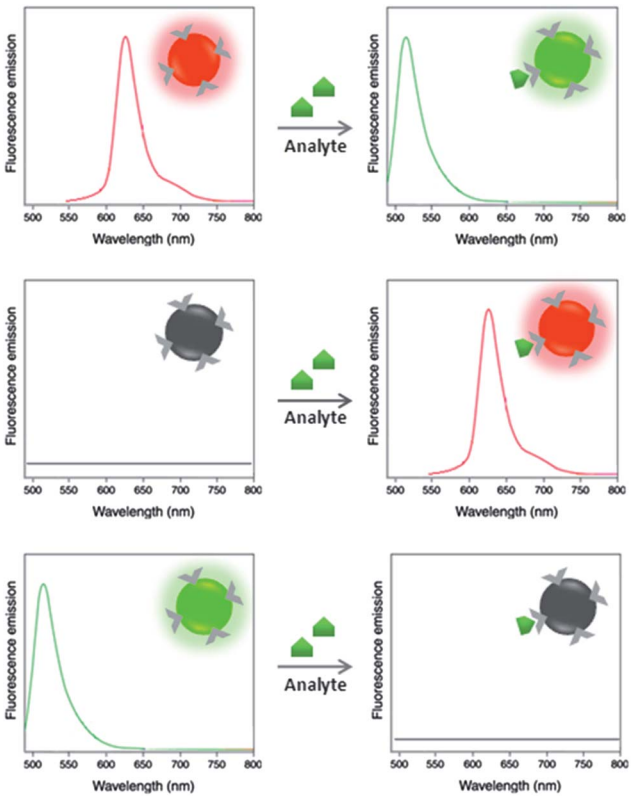



**Fig. 2** Types of spectral emission change upon biosensor–target interaction; (a) Stokes-shift wavelength change; (b) 'turn-on' fluorescence production; (c) 'turn-off' fluorescence quenching.

**Table 1** Protein and enzymatic markers characterised for the identification of biological fluids

| | |
|---|---|
| Blood | Glycophorin A,[1] haemoglobin[1] |
| Semen | Prostate specific antigen,[1] semenogelin[1] |
| Saliva | Human salivary amylase,[1] lysozyme,[1] mucin 7 (MUC7)[2] |
| Urine | Tamm-horsfall protein[1] |
| Sweat | Dermcidin (DCD)[32] |
| Vaginal secretion | Mucin 4 (MUC4),[1] human β-defensin 1 (HBD1)[2] |

Despite numerous suitable assets, the use of sensors as biological fluid identification strategies remains a relatively undeveloped field, with only a handful of sensors utilised in any aspect of criminal investigation (Table 2). This is likely due to construction challenges arising from a high dependency on target selection, necessitating a meticulous design and optimisation process for every individual assay. It is consequently prominent to explore a selection of sensors successfully employed within other analytical disciplines and examine their adaptability towards the detection of fluid-specific analytes.

## Environment-sensitive biosensing

One particular class of sensing molecules demonstrating potential use within fluid identification strategies is that of

**Table 2** Current use of fluorescent biosensors in various areas of forensic analysis

| | |
|---|---|
| Drug Detection | - Analysis of the 'date-rape' drug GBL in alcoholic drinks *via* static quenching sensors[33]<br>- Use of nucleic acid aptamers for identifying cocaine residues[34] |
| Explosives Detection | - Exploiting FRET changes in labelled proteins to detect nitroaromatic explosives[35]<br>- Intermolecular displacement of FRET quenching ligand by TNT-antibody binding[36] |
| Environmental Monitoring | - Screening of pathogens within drinking water through fluorescent biofilm sensing[37]<br>- Use of intramolecular substrate hydrolysis to confirm chemical toxin presence[38] |
| Fingerprint enhancement | - *In situ* enhancement of latent fingerprint residues *via* aptamer interaction[39] |

fluorescent molecules that display variable spectroscopic properties in response to changes in their local environment.[42] In particular, some of these fluorophores (solvatochromic fluorophores) are characterized by a very low quantum yield in aqueous solution, which markedly increases when exposed to a hydrophobic environment[43] (Fig. 3).

Such characteristics have found use in the monitoring of protein folding, in which an increase in the surrounding hydrophobicity of fluorophore-bound proteins resulted in fluorescence production upon transition from an unfolded to a folded state.[44] The same study was also able to modify these fluorophores so that they may be attached to peptide sequences using standard Fmoc chemistry.

Whilst this specific conjugation was used to detect an interaction between a labelled peptide and SH2 protein domains, application to the identification of biological fluids may be achieved through the attachment of modified fluorophores to biological moieties (*e.g.* peptides or oligos) able to bind fluid-specific proteins or enzymes. Upon interaction with an intra-fluidic target, the microenvironment surrounding the fluorophore will change, altering its emission properties to signify the analyte presence. For example, if binding between the biosensor and the analyte results in the formation of a hydrophobic microenvironment, the solvatochromic fluorophore will turn-on with a large enhancement of fluorescence. The feasibility of this approach for the detection of specific protein–protein interactions has already been demonstrated in several areas other than forensics.[45,46]

Despite demonstrating effective detection in many analytical disciplines, there are some challenges associated with this type of sensing. Target selection is increasingly difficult, with the most viable option being to conjugate probes to peptide or nucleic acid aptamer sequences known to bind specific targets.

Using combinatorial libraries, peptides and aptamers may be characterised that bind to molecules within blood, semen, and saliva. However once labelled, it is difficult to predict if such recognition moieties will retain previous target binding capacity. Resolving this issue may be aided through computation protein fold modelling. Furthermore, rigorous testing prior to *in situ* sensor implementation would be needed to examine the potential of false positives being generated by the wider environment of the scene itself.

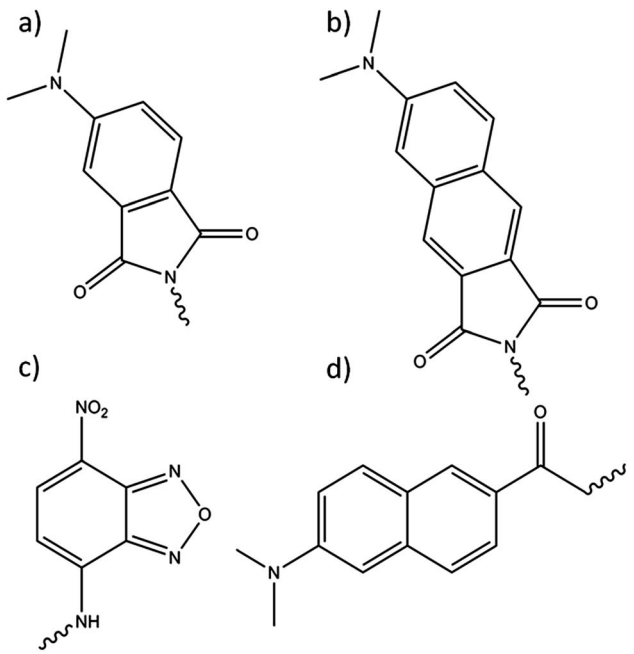



**Fig. 3** Sovatochromic fluorophores that can be applied for the development of environment-sensitive sensors. (a) 4DMAP (4-dimethylaminophthalimide); (b) 6DMN (6-dimethylaminonaphthalimide); (c) NBD (7-nitrobenz-2-oxa-1,3-diazole); (d) PRODAN (6-propionyl-2-(dimethylamino)naphthalene).

## FRET-Based biosensing

Förster Resonance Energy Transfer (FRET) refers to the phenomenon of non-radiative energy transfer between two fluorescent molecules as a result of electrostatic dipole–dipole interactions. This process principally culminates in the quenching of an excited donor fluorophore emission by alternative transmission to a proximate acceptor molecule, which is consequently allowed to fluoresce.

Energetic transfer is distance-dependent, with fluorophore separation greater than a length of approximately 10 nanometres alleviating quenching effects and allowing signal output as either simple donor emission observation or through the ratiometric measurement of an acceptor–donor spectral shift. FRET occurrence is often exploited within a number of macromolecular biosensing applications for monitoring biological interactions that facilitate a proximity change between two or more fluorescent molecules. FRET-based distance sensing occurs across an order of several nanometres and may therefore be considered ideal for the detection of small biomolecular elements.[47] Furthermore, whilst some FRET mechanisms may exploit the fluorescent properties of organic molecules themselves, the additional labelling of non-fluorescent recognition moieties with synthetic fluorescent dyes, allows the detection of analytes that would otherwise remain indiscernible. Two

distinct approaches are applied to the detection of molecular targets through FRET biosensing. The first involves the labelling of a sensing substrate with both donor and acceptor fluorophore moieties as a single intramolecular complex. Interaction with a testing analyte upon this complex alters the relative proximity of fluorophore molecules through the induction of a structural change within the sensor molecule.[40]

The second form of FRET sensing comprises fluorescent labels located upon two separate and independent biological elements, which subsequently unite as a result of binding interactions or disassociate *via* competitive displacement. The former of these may be considered impractical for the *in situ* identification of biological residues since it is largely impossible to label the initial testing antigen.

### Intramolecular FRET

Recent research has demonstrated the use of intramolecular FRET efficiency as a successful *in situ* assay for enzymatic activity, with previous studies utilizing catabolic events to monitor increased protease expression in both wound-infecting bacterial biofilms[48] and enteroviral pathogenesis *in situ*.[49] Testing is reliant upon the hydrolysis of a terminally labelled substrate conjoining two fluorophores in order to disrupt energy transfer processes (Fig. 4). As the specific cellular function of each body fluid necessitates unique enzyme production, similar turn-on fluorescence assays may be potentially developed for the *in situ* detection and identification of biological fluids.

A different but related approach involves the modification of enzymes themselves to serve as the fluorescently labelled sensors, undergoing a conformational change in the presence of a fluid-specific antigen. Such modifications have been exploited in a recent study in which the rotation of labelled F(o) F(1)-ATP synthase subunits allowed the detection of ATP synthesis and hydrolysis.[50] This assay successfully utilized the elastic structural deformations of the enzymatic protein backbone necessary for preliminary catalytic interaction with ADP in order to change the relative proximities of Alex-532 and Cy5 fluorescent dyes labelled upon three rotary enzyme subunits. However, this approach may be slightly more challenging for use within fluid identification, as it would require enzymes to be labelled at extremely specific sites.

The employment of intramolecular FRET sensing has been successfully integrated into oligonucleotide sequences capable of specific binding not only to other nucleotide sequences[51] but also to proteins[52] and inorganic molecules.[53] These assays employ a flexible dual-labelled oligonucleotide strand known as a 'molecular beacon', forming a self-complementing hairpin structure in order to bring donor and acceptor dyes within appropriate proximity for FRET quenching effects to occur. Subsequent hybridization or binding of the probe to the analyte causes a separation of the fluorophore moieties to successfully inhibit quenching and enhance fluorescence[51,52] (Fig. 4a). Recent research has demonstrated the *in situ* applicability of this technique for the detection of *Pseudomonas putida* bacteria within activated sludge and river water.[54]

In peptide beacons the oligonucleotide sequence is replaced by a peptide sequence with binding capabilities towards a specific target. Modifications to the flanking ends might be required in order to decrease the conformational freedom of the peptide sequence and allow the formation of a stable stem,[55] but the overall sensing mechanism remains the same as oligonucleotide molecular beacons. These peptide-sensing mechanisms have been developed for the detection of anti-HIV antibodies[56] and particular structural domains of enzymatic rennin[57] but have yet to demonstrate any pertinent *in situ* applicability. Recent advances in the use of combinatorial libraries now allow the selection of either oligonucleotides or peptides with binding capabilities towards virtually any target molecule. This suggests a great potential for these systems to be applied to forensic evidence recovery and analysis.

A further exploitable opportunity is provided by an intramolecular FRET mechanism occurring between a pair of fluorophores located at a specific distance on an antibody (Fig. 4 c). In this state, the fluorescence is at the emission wavelength of the donor, with limited fluorescence from the acceptor. The binding of the analyte of interest induces a conformational change in the structure of the antibody resulting in a change in proximity of two fluorophores, switching emission wavelength. Similar systems have been employed *in situ* for the detection of biochemical markers of myocardial infarction[58] and bacterial contamination in food products.[59] Antibodies make this type of sensor particularly suitable for body fluid detection. Antibodies are presently considered a gold standard in protein detection and would ensure high degrees of specificity towards fluid targets with the added value of species selectivity (*i.e.* human), characteristics that would be extremely beneficial in a forensic context.

### Intermolecular FRET

As previously mentioned, the use of intermolecular FRET processes within fluid-specific testing may be confined to

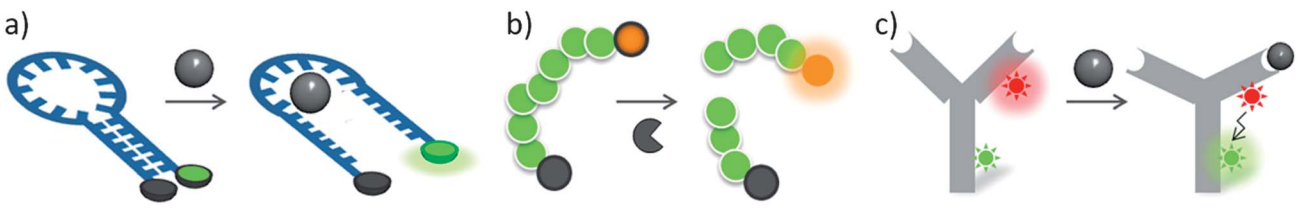



**Fig. 4** Demonstration of intramolecular FRET sensing mechanisms; (a) a fluorophore-quencher pair in an initially quenched oligonucleotide hairpin is separated upon binding, restoring fluorescence; (b) a terminally labelled fluorogenic substrate is digested by catalytic enzymes, releasing a fluorescent by-product; (c) analyte-induced conformational change upon antibody binding changes donor–acceptor proximity, resulting in a wavelength shift.

displacement only mechanisms, as it is largely impossible to pre-label unknown testing analytes. Development of a displacement-based FRET assay involves the preliminary integration of donor fluorophores upon a biomolecular sensing unit, which subsequently binds with moderate affinity to a non-testing substrate alternatively labelled with acceptor moieties, thereby permitting proximal FRET quenching. A positive sensing response occurs when the moderately bound quenching ligand is competitively removed in the presence of a macromolecular target with higher sensor binding affinity (Fig. 5).

The design of these assays permits a high degree of flexibility in the recognition moiety, allowing assays to be adapted accordingly to fulfill specific requirements. This mechanism also affords greater assay sensitivity, with the majority of individual sensing macromolecules allowing multiple donor fluorophore labelling, thereby enhancing signal output upon successful interaction.

Sensors for the detection of maltose have been designed on this principle, utilizing the dissociation of a quencher labelled β-cyclodextrin competitor from an *E. coli* maltose binding protein,[60] whilst other studies have produced similar success in the observation of mycotoxin-producing *amstelodami* fungal spores by displacing a moderately bound quencher-labeled fungal species from an anti-*Aspergillus* antibody complex.[61]

Other assays employ specific oligonucleotide sequences (*i.e.* aptamers) for fluorescent detection; DNA known to bind to a specific analyte is labelled with a fluorophore and subsequently hybridized to a complementary sequence bearing a quencher molecule, making the system non-fluorescent. The assay exploits conformational changes that aptamers undergo upon target interaction, which displaces the complementary quenching strand and restores the fluorescent signal (Fig. 5 a).

These assays show high relevance to the detection of biological fluid depositions, with modification likely focusing on replacing current antibody and aptamer elements with those able to bind intra-fluidic molecules. Regardless of the numerable benefits displayed by FRET-displacement based technology, evaluation into the applicability of this technique towards any biomedical or industrial *in situ* analysis seems to remain currently unexplored.

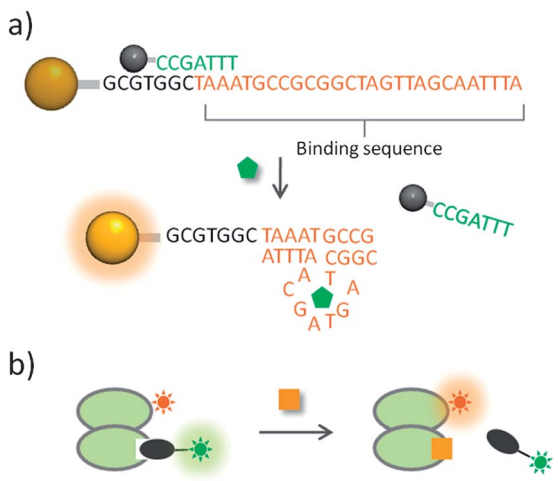



**Fig. 5** Examples of varying intermolecular FRET transductions; (a) a fluorophore labelled oligonucleotide sequence is initially hybridized to a complementary quenching strand which is subsequently displaced by a target analyte, producing fluorescence; (b) moderately bound acceptor ligands are displaced *via* a higher affinity competitive analyte.

## Static quenching-based biosensing

Since energetic transfer occurs whilst the donor molecule exists within an excited state, the phenomenon of FRET may be described as a 'dynamic' quenching mechanism. Conversely a number of processes also exist based on the phenomenon of 'static quenching', in which two fluorophores form a dimeric ground-state intermolecular complex. Physical contact between the fluorescent moieties results in the creation of an entirely new excitation absorption spectrum, thereby inhibiting fluorescence emission.[62] This effect is particularly prevalent when binding occurs between two identical fluorophores in the formation of homodimers.

Within free solution fluorophores may aggregate of their own volition due to hydrophobic, electrostatic or steric forces,[63] however the high fluorescence contribution from remaining unbound dye molecules often renders the total reduction in fluorescence intensity negligible.[64] Substantial or even total quenching arises from the integration of the fluorophore moieties into a particular macromolecular substrate with a significantly high degree of substitution as to force the majority of dye molecules into dimeric quenching complexes.[65]

Efficient substrate-based quenching *via* homodimeric binding interactions has been utilized in the recent development of analytical biosensors for enzymatic activity. Sensing depends on the initial labelling of numerous quenching dye moieties in a heavy stoichiometric ratio upon an individual substrate molecule, with consequent digestion of the substrate backbone by enzymatic activity allowing dissociation of dimeric complexes[65] (Fig. 6). Although the dimerization of all fluorescent molecules will result in the diminishment of fluorescence emission, these assays customarily utilize rhodamine or BODIPY derivatives as a result of increased aggregation rates compared to other organic dyes.[63] Previous studies employing these substrate-confined dyes have successfully demonstrated the *in situ* detection of elastase activity associated with dental pellicle production by salivary glycoproteins,[66] as well as the proteolytic action of calpain enzymes accompanying cataract formation in sheep.[67]

The modification of previous *in situ* assay technologies for application to the differentiation of biological fluid depositions would largely revolve around the development of a substrate capable of binding, and subsequently separating, a large number of dimerized fluorophores upon interaction with a fluid-specific enzyme. Suitable sensing substrates may comprise any biological macromolecule that can be successfully derivatized to include a considerable number of exposed amine binding groups for conjugation with organic dyes *via* simple covalent chemistry.

The use of fluorescent conjugated polymers (CPs) may also offer appropriate static-quenching based biosensors for fluid

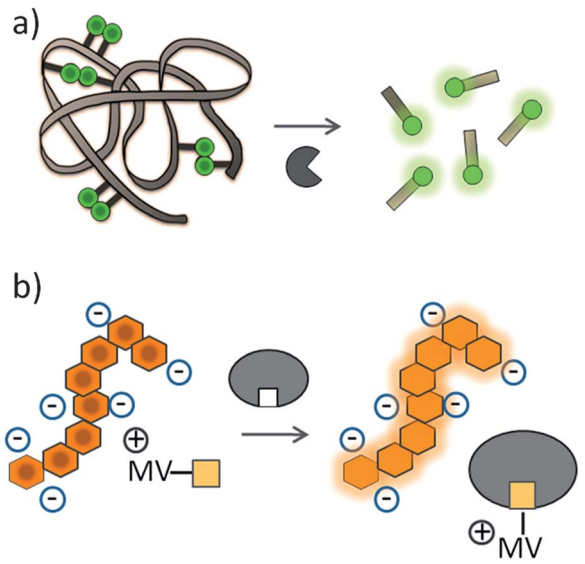



**Fig. 6** Static quenching based sensors; (a) the liberation of dimerized fluorophores from a heavily labelled backbone by digestive enzymes; (b) the recognition moiety is conjugated to methyl viologen ($MV^{2+}$), which is electrostatically attracted to the CP, therefore quenching fluorescence. In the presence of the analyte, the recognition moiety and therefore the $MV^{2+}$ are pulled away from the CP, restoring fluorescence.

detection purposes. It has been demonstrated that polyanionic conjugated polymers can be quenched by cationic electron acceptors in aqueous solutions. By conjugating biotin to the cationic acceptor and allowing interaction to take place between the CP and the conjugate, complete quenching of the polymer fluorescence can be achieved.[68] In the presence of streptadivin, binding to the biotin molecule ligand takes place, displacing the quencher from the polymer, restoring its fluorescence. Adapting this assay to identify fluid-specific molecules would require the quencher to be linked to an antibody or binding protein; this complex can then be removed from the polymer by interaction with a fluid biomarker. What makes the interaction between CPs and cationic acceptors advantageous over dynamic FRET-based sensing for *in situ* target detection is the absence of associated background fluorescence, allowing the localization of discrete fluid deposition areas.

## Conclusions

A number of biosensing mechanisms demonstrating a potential application to the detection and identification of biological fluids have been outlined. These assays have established value in many comparable *in situ* applications and show high adaptability to the sensing of fluid-specific targets. Successful sensor application is likely to provide numerous advantages over current presumptive testing methodologies.

Biosensors are often noted for their high target sensitivities, with many recognition moieties able to detect biomolecules at extremely low concentrations within complex matrices. This may be particularly prominent in the detection of enzymatic units, which react with multiple sensing substrates and generate a cumulative fluorescence effect. However, successful detection of a positive signal response is primarily dependent upon the sensitivity of the detection equipment itself, which must be taken into account during sensor design.

Depending on the recognition element utilised, biosensors also offer an extremely high molecular specificity, with transduction arising from a wide array of biological activities, including highly exclusive antibody and protein complex affinity interactions. This level of specificity not only allows differentiation between fluid sample types but may also distinguish human tissues from their non-human counterparts.

Furthermore, assays may be individually customized for optimal fluorescence emission, with an array of nanoparticles, organic dyes, solvatochromic fluorophores and conjugated fluorescent polymers available to produce a tailored signal output. This not only results in increased assay sensitivity through higher quantum yields and fluorescence lifetimes but gives potential for the simultaneous detection of several inter-fluid analytes within a single multiplex application by exploiting variable wavelengths.

The process of applying biosensors to fluid depositions within a forensic context is also likely to be very simple, with application of sensing molecules to evidential surfaces achieved *via* spray dispersal. Despite many studies, including those conducted within our own research group (unpublished data), proving the efficacy of topical spray application in a select number of biosensors,[69] the physical challenges of spraying individual sensor constructs must be recognized. It is therefore advised that rigorous *in situ* testing is conducted for each sensor design prior to application. This is equally important when considering sensor stability. Whilst many sensors, once assembled, remain stable for an extended period, each specific sensor will likely have a strict set of environmental conditions required to avoid degradation. Stability challenges must therefore be addressed on a sensor-by-sensor basis.

Despite a myriad of potential *in situ* detection benefits, biosensor use for the identification of biological fluids remains exclusively designed for the screening of analytes within solution, employing spectrofluorimetry in order to measure the fluorescence output. It is therefore conducive that a selection of biosensing mechanisms be tested in order to demonstrate proof-of-concept towards *in situ* analyte detection prior to modification of validated techniques for interaction with fluid-specific biomolecules.

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