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SHORT COMMUNICATION

# Development of a biosensor for human blood: new routes to body fluid identification

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**Abstract** The identification of human blood at a crime scene can provide crucial information to an investigation whilst also providing a source of nuclear material which can be targeted for DNA profiling. Here, we report on the development of an immunofluorescent biosensor for the identification of human blood which has the potential to overcome the drawbacks of the current body fluid identification techniques. An antibody (Ab) raised against human erythrocytes was conjugated to fluorescent semiconductor quantum dots (QDs) by sulfhydryl chemistry. The conjugation was verified by agarose gel electrophoresis and immunohistochemistry. Incubation of liquid blood samples with the conjugated nanocrystals was shown to quench the fluorescence emission spectra in a concentration-dependent manner. A different effect was observed with unconjugated QDs incubated in blood. Full profiles were obtained from blood samples previously treated with the Ab–QDs, demonstrating that the method does not interfere with DNA profiling. To our knowledge, this is the first example of a hybrid Ab–QD sensor that has the potential to be employed for the identification of human blood. The results of this study are expected to open up a new research direction in the field of body fluid detection.

**Keywords** Anti-glycophorin A · Biosensor · Erythrocytes · Forensic · Immunofluorescence · Quantum dots

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## Introduction

The identification and localization of body fluids at a crime scene can indicate the nature of the activity involved and the severity of a crime and can also specify the area that should be targeted for DNA profiling. This is particularly true for blood, which represents the most common and valuable type of evidence encountered in forensic investigations. The ideal body fluid identification technique should be rapid, inexpensive, sensitive, human-specific and non-destructive to the target fluid in order to preserve the DNA. During scene examinations, forensic examiners initially carry out a visual examination to locate potential stains; this is then followed by presumptive tests that provide a preliminary indication of the nature of any stains located. These presumptive tests have several drawbacks in terms of sensitivity, specificity and DNA recovery. Often, additional tests are required to confirm both the identity and the origin of a stain. Such analysis, besides being laborious and costly, could delay police investigations and interfere with the solving of a case. Moreover, fluid stains are not always visible to the naked eye, and the currently available tests cannot be used to locate them, carrying the risk of missing crucial evidence.

Recent advances in the area [1] established proof of concept for the in situ detection and localization of human blood using fluorescently labelled, human-specific antibodies. However, the procedure is unsuitable for implementation at crime

scenes due to the weak fluorescence of organic dyes as well as the technical difficulty of removing the unbound antibody after the solution is applied to forensic substrates.

The introduction of quantum dots (QD) as biosensors has the potential to overcome this limitation. QDs exhibit large extinction coefficients, intense brightness, high photostability and resistance to photobleaching, making them superior to organic dyes in every way [2]. QDs are thus gaining prominence as the 'next-generation fluorophores' and are being used in diverse applications. Moreover, functional groups added to the QD surface permit conjugation to biomolecules like Abs, proteins and aptamers [3, 4]. Conjugation of antibodies to QDs is an ideal means to target antigens in applications demanding high specificity. Furthermore, the binding of an antibody-conjugated QD (Ab–QD) to its target antigen can affect the local environment of the QD and induces an immediate visible change in the fluorescence, making it ideal for a biosensor as this precludes the need of a wash step. An example is given by the work of Dwarakanath et al. [5]; the authors described a detection method based on a 'shift' in emission wavelength upon incubation of aptamer– or Ab–QDs with bacteria. They reported the appearance of a peak between 60 and 140 nm away from the wavelength of the expected emission peak and attributed it to a decrease in QD size upon binding to the target. Additionally, a significant quenching effect of the QD emission peak was observed. Similarly, other studies have reported changes in emission wavelength when functionalised QDs interact with the target. Carillo-Carrion et al. [6] used Colistin-conjugated QDs to detect *Escherichia coli* and observed both a blue shift in emission wavelength as well as a quenching effect which was proportional to the bacterial concentration. A study aimed at the detection of the explosive trinitrotoluene (TNT) found a strong quenching effect of the QD fluorescence in the presence of TNT [7]. The author proposed either a charge transfer or the formation of new trap states as the likely mechanism. Riegler et al. [8] also observed an unexplained blue shift in wavelength when five conjugated QDs were combined in a multiplex. An energy transfer process upon QD binding to the target was suggested to be the cause. On the other hand, when aptamer-conjugated QDs bound to the target bacterial spores, a proportional enhancement of fluorescence intensity was recorded [9].

The development of biosensors has had a great impact in a number of research areas in which specific identification and detection of an analyte is essential. However, the field has not fully made its way into forensic science area even though it is considered the detection science *par excellence*. An Ab–QD conjugate which exhibits specific changes in fluorescence only upon binding to the target antigen would in fact be an ideal forensic biosensor. In the present study, anti-glycophorin A was selected as the antibody of choice because it exhibits a strong affinity to human glycophorin A, a glycosylated protein present in abundance on the membrane of erythrocytes [10]. Our study has previously demonstrated the species specificity of this antibody [1, 11]. In the current work, we exploited the specificity of this antibody together with the properties of QDs towards the development of a sensor that would allow the detection of human blood. The preparation of glycophorin A Ab–QD conjugates and their ability to be used as a rapid and efficient identification method are described. The possibility to further develop such a system for the localization of body fluids deposited on forensic relevant substrates is also discussed.

## Materials and methods

### Chemicals, equipment and sources

Pre-cleaned glass microscope slides (25 × 75 × 1 mm) were purchased from Thermo Scientific (UK); mouse anti-human glycophorin A monoclonal antibody (HIR2) was supplied by Abcam (Cambridge, UK). The eFluor® 605 Nanocrystal Conjugation Kit–Sulfhydryl Reactive (eBioscience, San Diego, CA) was used to perform the conjugation reaction. E-Fluor® 605 Nanocrystal are CdSe/ZnS quantum dots functionalized with polyethylene glycol (PEG) on the outer surface for the conjugation to the sulfhydryl moieties present on the antibodies. Quantum dot concentrations and labelling ratios were measured on a ND-1000 Nanodrop spectrophotometer (LabTech International, Ringmer, UK). Fluorescent images were acquired on a Zeiss Axioskop 2 MOT plus stereomicroscope (Carl Zeiss Ltd., Welwyn Garden City, UK) and imaged using a TCS SP2 system. Images were captured with an AxioCam HRC CCD camera and processed using Axiovision 3.1 software.

The fluorescence emission spectra were measured on a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) using 325-nm excitation wavelength to scan the emission spectrum. Cary Eclipse software was used for fluorescence data collection.

For DNA profiling, samples were quantified in a 96-well plate on the ABI prism 7000 (Applied Biosystems). DNA was then amplified using a GeneAmp PCR System 9700 PCR machine (Applied Biosystems). Capillary electrophoresis was run on the ABI PRISM® 310 Genetic Analyser and evaluated using GeneScan® 3.1.

### Sample collection and storage

Human blood was collected from healthy volunteers after informed consent. Blood was drawn by venipuncture using BD Vacutainer® Safety-Lok™ Blood Collection Sets

(Becton Dickinson, Oxford, UK); 10 mL was collected in sterile blood vacutainers containing 7.5 % EDTA (Becton Dickinson). All blood tubes were stored at 4 °C until further analysis.

### Conjugation of antibody to quantum dot nanocrystals

The conjugation procedure by sulfhydryl chemistry was performed using the protocol specified in the eFluor® 605 Nanocrystal Conjugation Kit, with minor modifications. Briefly, the antibody was directly incubated with the QDs for 2 h in the presence of a reducing agent for the formation of a thioether bond. At this stage, the protocol was modified for use with 100 μg of antibody. The reaction was terminated by the addition of a quencher for 10 min. The mixture was then washed by spin filtration using a 100k molecular weight cutoff filter to separate out the conjugated product. The final eFluor® nanocrystal antibody conjugates (Ab–QDs) were stored at 4 °C in the dark.

### Agarose gel electrophoresis

The conjugation product was verified by agarose gel electrophoresis using agarose, routine use (Sigma Aldrich, UK) and Tris-borate–EDTA buffer (TBE) 10× stock solution (Severn Biotech Ltd.). Gels were prepared at 0.5, 2 and 3 % concentrations and run using a GPS 200/400 Electrophoresis Power Supply (LKB/Pharmacia, Sweden) with 0.5× TBE as the running buffer at 90 V for up to 3 h at 6 °C.

### Fluorescence microscopy

Immunocytochemistry was performed using Ab–QDs on freshly prepared blood smears. Three microlitres of blood was smeared onto pre-cleaned microscope slides and allowed to air dry for 30 min. The smears were spray-fixed in methanol/acetone (70:30, *v*/*v*) at room temperature and allowed to dry for 10 min. A small area of each slide was then defined for antibody labelling by marking a circle on the undersurface of the slide using a permanent marker. The performance of two different blocking steps was evaluated (refer to Electronic supplementary material (ESM) S3 and Table S1). The Ab–QD conjugates were directly applied to the smear and incubated for 30 min at room temperature. The unbound conjugates were removed by washing in PBS twice for 2 min. Both positive and negative controls were included (refer to ESM S2).

### Blood assay using Ab–QDs

The Ab–QD conjugates (10 nM) were directly added to undiluted or diluted blood samples (total volume, 200 μL) and incubated at room temperature for 30 min. The fluorescence spectrum (350–620 nm) was recorded for the following samples: whole blood, Ab–QDs in PBS, conjugated Ab–QDs and unconjugated QDs in blood. The peak intensities were normalized by calculating them as a percentage of the original fluorescence. To compare the changes in peak intensity before and after incubation of Ab–QDs in blood, a *t* test was performed on the peak intensity at 605 nm. Statistical analyses were carried out using the SPSS® 17 (SPSS Inc.) software package.

### DNA profiling

Whole blood samples from two independent donors were subjected to DNA profiling using SGM Plus. Ab–QDs were added to 20 μL of blood in triplicate to a final concentration of 10 nM based on the concentrations used for the spectrofluorimeter measurements. The DNA was extracted, quantified by real-time PCR (Quanitfiler®) and amplified using 28 cycles in a 25-μL final reaction volume. Positive controls (untreated blood samples) were included.

## Results and discussion

### Verification of antibody–nanocrystal (Ab–QD) conjugation

Confirmation of the Ab–QD conjugate formation was carried out before proceeding with further experiments. An entire QD molecule with all layers has a molecular weight of approximately 200 kDa, whilst a single IgG antibody molecule has a reported weight of 150 kDa. Therefore, the conjugation procedure QD can accommodate the attachment of two antibodies to a single nanocrystal. The difference in weight between unconjugated nanocrystals (NCs, 200 kDa) and conjugated NCs (350–500 kDa) would be expected to exhibit a corresponding difference in the electric field drift and migration through the gel. Three concentrations of the

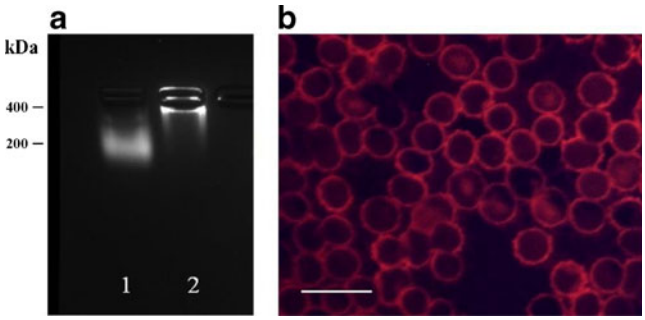


**Fig. 1** **a** Products of the sulfhydryl conjugation (*lane 2*) were run on a 3 % agarose gel along with unconjugated QDs (*lane 1*) as a control. **b** Erythrocytes incubated with Ab–QDs showed staining specific to the membrane. *Scale bar*, 10 μm

agarose gel were tested. The sulfhydryl Ab–NCs, due to the larger size, appear to migrate only within the 0.5 and 2 % gel (data not shown), but remain in the sample wells of the 3 % gel, even after 3 h (Fig. 1a). The effect of gel concentration on migration was taken as an indication of a size difference between the conjugated and unconjugated QDs. As a further confirmation, immunocytochemistry was also employed. Human blood smears incubated with 10 nM Ab–QDs from the sulfhydryl conjugation are shown in Fig.1b. The staining was specific to the erythrocyte membrane and was not observed with either of the negative controls, unconjugated QDs on blood, or Ab–QDs in the absence of blood (refer to ESM S2).

The results of agarose gel electrophoresis and immunocytochemistry provided positive confirmation that antibody conjugation to the QD was achieved, that the antibody was capable of actively interacting with the target antigen and that the conjugation did not adversely affect its binding or specificity. Moreover, conjugation to the Abs did not affect the fluorescence emission of the QDs (refer to ESM S1).

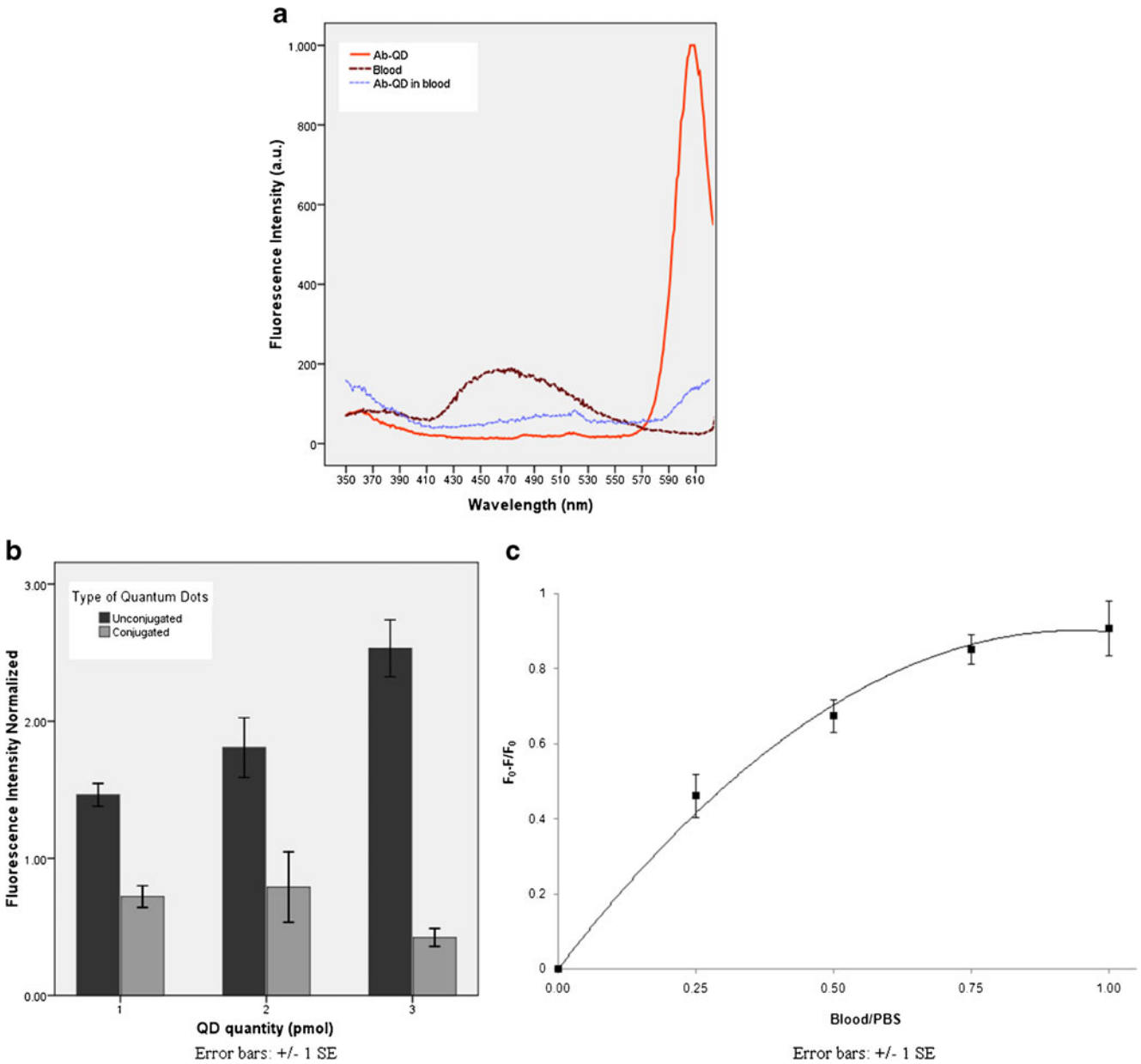


**Fig. 2** **a** Fluorescence profiles of Ab–QDs and blood indicating the two main peaks of interest: the plasma peak of blood at 460 nm, the Ab–QD peak at 605 nm and the quenching effect observed after incubation of Ab–QDs in blood. **b** Relative peak intensities (normalized) at 605 nm of the sulfhydryl Ab–QDs at different concentrations in comparison to unconjugated QDs. **c** Decrease in the fluorescent signal at 605 nm as a function of the concentration of blood. The curve was fitted to a quadratic polynomial equation; *error bars* indicate the standard deviation of three different assays

## Reaction of Ab–QDs with the target antigen in blood

When Ab–QDs were incubated for 30 min in undiluted liquid blood samples, the following two features of interest were recorded in the fluorescence emission spectrum (Fig. 2a). The Ab–QDs alone (concentration, 10 nM) emit a narrow peak at 605 nm. The intensity of this peak drops by approximately 90 % of its original intensity in the presence of blood, which strongly quenches the QD fluorescence. Furthermore, a quenching effect of the plasma peak at 460 nm was observed after incubation with Ab–QDs. The normalized spectrum of the plasma peak at 460 nm shows a decrease in intensity by 52.58 % ($t$=21.45, $p$=0.001, one-tailed). The degree of quenching at 605 nm with Ab–QDs in blood instead of PBS was more pronounced. A $t$ test of the peak intensity showed quenching of fluorescence by 92.26 % ($t$=113.04, $p$<0.001, one-tailed); this feature was therefore the focus of subsequent experiments. Moreover, the incubation of Ab–QD conjugates with semen and saliva, the other two most common body fluids encountered at crime scenes, did not result in the quenching effect observed with blood (data not shown).

When different concentrations of both Ab–QDs and unconjugated QDs were incubated in blood and compared, the results obtained were remarkably different. At 605 nm, an increase in unconjugated QD concentration produces a corresponding increase in peak intensity, as expected, whilst the Ab–QDs show no significant change in peak intensity (Fig. 2b). A one-way ANOVA on the peak intensity was significant for the difference between conjugated and unconjugated QDs ($F$=85.34, $p$<0.001) as well as for the interaction between the type of conjugate and the QD concentration added ($F$=17.43, $p$=0.001).

The quenching effect is independent of Ab–QD concentration, resulting in a pattern that is specific to the antibody present on the QDs. There appears to be a greater interaction between the Ab–QD and the target antigen on the erythrocyte membrane such that any further addition of Ab–QDs to blood does not result in significant changes to the fluorescence intensity. In contrast, an increase in the concentration of unconjugated QD results in an increased fluorescence. This is likely due to the lack of interaction between the QDs and blood, which allows the QD fluorescence to increase with increasing concentration.

## Effect of antigen concentration on fluorescence intensity

Different dilutions of blood (0, 25, 50, 75 and 100 %) made in PBS were also tested with the conjugated QDs. As the concentration of blood increased, the quenching was also shown to significantly increase (Fig. 2c; one-way ANOVA: $F$=42.89, $p$<0.001). The quenching effect appears to be strictly correlated to the number of cells present in solution and, as a consequence, to the number of antigen molecules. When the Ab–QD is exposed to glycophorin A on the erythrocyte membrane, the binding brings the QD in close proximity to the cell, likely resulting in an energy transfer process from the Ab–QD conjugate and in the quenching of its fluorescence.

Energy transfer is highly sensitive to the distance between the fluorescence donor (QD) and the acceptor (target) [12, 13]. The multiple surface modifications of the QD, such as functionalization, PEGylation and conjugation to antibodies, increase the separation distance between the QD and the target antigen. However, the significant flexibility of the lipid–PEG layer allows for a spatial arrangement of the formed complex that results in efficient energy transfer processes [14] mirrored by the quenching effect observed when the Ab–QD binds to the target antigen in blood.

Avenues for reducing the intermolecular distance and optimizing the angle of orientation of the antibody could be explored to further enhance the quenching effect. This could include a conjugation chemistry using EDC, which is a zero-length cross-linker that provides the shortest possible distance. Another method would be to fragment the antibody to include just the antigen-binding (Fab) region, as demonstrated by Barat et al. [15].

Whilst the quenching mechanism cannot be fully explained, we believe that the effect observed upon binding of the Ab–QDs to the erythrocytes could be further explored in a viable biosensing application. Although similar effects have been previously reported [6, 8, 16], the precise relationship varies with the nature of the interacting systems involved, whether antibodies, nucleic acids or peptides. In order to further exploit the phenomenon of quenching or wavelength change upon target binding, the causation and mechanism must be clearly elucidated.

## Effect of Ab–QDs on DNA profiling

The application of Ab–QDs as biosensors to detect human blood would be of limited value if the QDs themselves inhibited

**Table 1** Effect of Ab–QD conjugates on DNA profiling

| Sample ID | No. of alleles amplified | Amplified DNA quantity (ng/μL) |
|---|---|---|
| Control 1 | 22 | 2.28 |
| Sample 1a | 22 | 2.46 |
| Sample 1b | 22 | 2.53 |
| Sample 1c | 22 | 4.11 |
| Control 2 | 22 | 3.08 |
| Sample 2a | 22 | 4.21 |
| Sample 2b | 22 | 3.1 |
| Sample 2c | 22 | 3.7 |

DNA profiling results of samples treated with Ab–QD conjugates (samples 1a, b, c and sample 2a, b, c) compared with untreated samples (controls 1 and 2)

subsequent DNA profiling stages. Locus dropout was assessed by expressing the results as the number of alleles obtained using SGMPlus™ (out of a total of 22; Table 1) and by the concentration of DNA in each sample obtained by real-time PCR.

No inhibition could be detected when blood samples treated with the Ab–QD conjugates were compared to the control samples (untreated blood) and the alleles at each of the 16 loci were all identified. Full profiles were obtained in all cases, and the resultant electropherograms from both control samples and treated samples were of indistinguishable quality (data not shown). It was also observed that in the quantification stage prior to PCR amplification, treatment with Ab–QDs did not interfere with the PCR, as proven by the successful amplification of internal PCR controls in the real-time PCR quantification process (IPC, Quantifiler™ Internal PCR Control, Applied Biosystems; refer to ESM S4).

## Conclusion

We have demonstrated an Ab–QD-mediated detection of liquid blood using the changes to fluorescence profiles caused by a specific bio-recognition reaction. The test developed is straightforward and rapid; specialized equipment or trained personnel would not be required to conduct the analyses. The use of a highly specific antibody as the recognition moiety would make the test confirmatory in nature. This, together with the possibility to obtain DNA profiles directly from the treated samples with no interference, would remarkably benefit investigations in terms of time and cost. If proof of concept can similarly be established for the detection of dried blood on a variety of surfaces, the success of this novel approach would pave the way for the development of a Ab–QD solution that could be sprayed as an aerosol onto a target surface for the detection of human erythrocytes in situ. A fluorescence surface scanner could be employed to measure changes in the signal intensity at a specific wavelength. The quenching effect can be potentially incorporated into a biosensing mechanism by two steps. First, a spectral scan of the target surface is required before incubation with the Ab–NCs. Upon incubation with the Ab–QDs, quenching of the florescence would provide stronger evidence of the presence of blood. Any blood present would manifest as a characteristic change in the fluorescence emission profile in comparison with the surrounding surface, which could indicate the presence and/or location of blood. Future directions for this study would be to extrapolate this technique where Ab–QDs can be developed to target other human-specific body fluid markers, and be tested for its potential capability as a multiplex of different Ab–QDs, such that simultaneous detection of several human body fluids could be achieved.

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