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Monitoring Criminal Activity through Invisible Fluorescent ‘Peptide Coding’ Taggants.

James Gooch,^a Hillary Goh,^b Barbara Daniel,^a Vincenzo Abbate^c and Nunzianda Frascione^{a,*}

^aAnalytical and Environmental Sciences Division. King’s College London. 150 Stamford Street. London, UK. SE1 9NH.

^bDepartment of Biological Sciences. National University of Singapore. 14 Science Drive 4. Singapore. 117543.

^cInstitute of Pharmaceutical Science. King’s College London. 150 Stamford Street. London, UK. SE1 9NH.

ABSTRACT: Complementing the demand for effective crime reduction measures are the increasing availability of commercial forensic ‘taggants’, which may be used to physically mark an object in order to make it uniquely identifiable. This study explores the use of a novel ‘peptide coding’ reagents to establish evidence of contact transfer during criminal activity. The reagent, containing a fluorophore dispersed within an oil-based medium, also includes a unique synthetic peptide sequence that acts as a traceable “code” to identify the origin of the taggant. The reagent is detectable through its fluorescent properties, which then allows the peptide to be recovered by swabbing and extracted for ESI-MS analysis via a simple liquid-liquid extraction procedure. The performance of the reagent in variable conditions that mimic the limits of a real world use are investigated.

Continuing efforts to reduce the substantial costs of crime on society have in recent years produced a number of highly effective criminal prevention technologies. The invention of forensic ‘taggants’ is a successful example of such advances. Taggants are defined as materials that may act as a means of physically or chemically marking an item in a way that allows it to become uniquely identifiable.¹ Usually consisting of three molecular components, taggants will include: a ‘coding’ element (to infer uniqueness in the taggant), a system that allows the taggant to be visually detected and a medium where these molecules are dispersed.

There are largely four forensic purposes for such materials. The first attempts to combat counterfeiting activities via the addition of a taggant to products (medicines, bank notes, official documents, etc.) to signify that they are of a genuine origin.² The second aims at marking valuable property (vehicles, electronic items) to associate them with a particular owner and discourage theft.³ The third involves the surreptitious addition of taggants into illicit substances (illegal drugs, explosives), so that their distribution or use may be monitored by law enforcement agencies.⁴ The last comprises the covert transfer of taggants to individuals during criminal activity in order to link them to a specific offence (e.g. handling firearms, trespassing).⁵ The physical nature of a taggant reagent will be highly dependent on which of the above purposes it is required. For example, taggants employed in covert operations should be invisible to the naked eye, to avoid undesired detection by the offenders³. The medium of each taggant will also vary depending on whether it needs to be easily transferred to an individual or remain anchored upon the product it is marking³. Only the coding element remains largely consistent across all tagging purposes.

One of the most popular methods to obtain this taggant coding is through the inclusion of unique DNA markers. Companies such as Applied DNA Sciences, Obiex and SelectaDNA produce taggants containing either botanically derived or syn-

thetic DNA oligonucleotides. The specific sequence of these oligonucleotides is then registered to a particular owner via an internal database.⁶ When required, the taggant may be recovered to extract and ‘match’ DNA to the owner’s information. Whilst DNA taggants have been shown to decrease the number of Cash and Valuables in Transit (CViT) offences,⁶ limitations in their analysis have been identified. The extraction, amplification and sequencing methods employed to isolate and match DNA are expensive and time consuming. Moreover, the labile nature of DNA has raised significant concerns over the general stability of DNA taggants⁷. Research into their use in monitoring fuel adulteration determined that short sequences of DNA within taggants, had poor stability and were often susceptible to erroneous identification⁸.

Another recently emerging method of encoding focuses on the use of varying trace elements to identify taggants. Reagents produced by the UK manufacturer Smartwater, contain a number of rare elements that are combined in unique formulations, allowing each taggant to be registered against a specific owner ID. After a reagent is detected (via the use of an incorporated fluorescent dye), laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is used to determine the individual formulation of the taggant and the owner that it is registered to (once again via internal database)¹. However, much like DNA-based coding systems, these tracer taggants are once again limited in their analysis. The use of multiple elements within a single taggant carries the potential of the formulation being incompletely recovered, which could then lead to erroneous identification. This problem stems from the differing transfer rates and stability of each individual tracer component, resulting from their chemical differences.

The development of a forensic taggant that is stable and can be rapidly and completely recovered is therefore critical to advancing crime reduction. In light of this, our research group recently suggested a novel ‘peptide coding’ reagent (or peptide taggant) as an alternative to inefficient taggant analysis

procedures⁹. This reagent has been designed to be covert and transferrable so that it may be used to mark an individual or object during criminal activity without the knowledge of the offender. Like the majority of taggants, our reagent comprises three distinct components. The base medium consists of a mixed hydrocarbon formulation (MHF) which is easily transferred to persons or clothing. Detection is achieved by Rhodamine-110 fluorophores, added in such a concentration as to make the taggant invisible under normal lighting conditions but highly fluorescent upon excitation by an appropriate light source. The specificity of this reagent (and main advantage over currently available taggants) is derived from the insertion of a unique peptide, where the amino acid sequence acts as an identifiable code for that particular taggant. The wide variety of natural and unnatural amino acids available ensures that the peptide may be sufficiently individual, without extensive length. Once the reagent is transferred as a result of criminal activity, it may be easily and rapidly recovered. First, areas of taggant presence may be indicated through fluorescence excitation, allowing complete recovery of the reagent by targeted swabbing. The peptide may then be isolated from the oil-based medium and swab via simple liquid-liquid extraction. The mass-to-charge ratio of the amino acid sequence is then revealed via Electrospray Ionization Mass Spectrometry (ESI-MS), identifying the particular taggant.

Whilst developed as an initial proof of concept, this taggant may have the potential to be used within criminal investigation to establish evidence of primary (object-to-person); and possibly even secondary (object-to-person-to-object) contact transfer. This work therefore involves a comprehensive investigation into the ability of peptide coding reagents to operate effectively within a real-world setting. Significant examination of the transfer, detection, recovery and stability of multiple peptide reagents was undertaken to demonstrate their advantages over currently available forensic taggants.

EXPERIMENTAL SECTION

Peptide Synthesis

Peptides Ac-KQQSP-NH₂ (Sequence 1), Ac-HSSKL-COOH (Sequence 2), and Ac-hsskl-COOH (Sequence 3) were manually synthesized by Fmoc-Solid Phase Peptide Synthesis. NovaPEG rink amide (Merck Chemicals Ltd, UK) and 2-chlorotriethylchloride (Sigma Aldrich, UK) resins were used to prepare sequences 1 and 2-3 respectively. Resins were swollen in dimethylformamide (DMF) inside empty fritted polypropylene tubes for 30 minutes prior to coupling. All amino acid coupling steps for sequence 1 were performed using 4 equivalents of Fmoc amino acids with N,N'-Diisopropylcarbodiimide (DIC) and Ethyl (hydroxyimino)cyanoacetate (Oxyma) in DMF in a molar ratio of 1:1:1 of amino acid, DIC and Oxyma, respectively. The first coupling step for sequences 2-3 was performed using 1 equivalent of Fmoc amino acid with 5 equivalents of N,N'-Diisopropylethylamine (DIPEA) in dichloromethane (DCM). Subsequent coupling steps were performed using the same procedure utilised in the assembly of Sequence 1. Coupling reactions were allowed to take place for 1 hour with successful conjugation monitored by the picrylsulphonic acid test. After coupling, Fmoc de-protection of all amino acids was achieved via treatment with 20% (v/v) piperidine in DMF for 30 minutes and confirmed by the picrylsulphonic acid test. Impurities and excess reagents were removed at each stage via multiple washes using DCM, and DMF. The N-terminus of each

peptide was acetylated using ten equivalents of acetic anhydride and twenty equivalents of DIPEA in DMF for 1 hour. Resins were lastly treated with a solution of Trifluoroacetic acid (TFA): distilled water: phenol: thioanisole (TA): triisopropylsilane (TIPS): 2,2'-(Ethylenedioxy)diethanethiol in a 90:5:5:5:2.5:2.5 molar ratio for 3 hours at room temperature to remove the completed peptides from the solid support. The filtrate was then concentrated under nitrogen at 40 °C before the addition of 10 equivalents of ice cold diethyl ether to precipitate the peptides. The wash and precipitation process was repeated several times before the final obtained peptide pellets were freeze-dried for 24 hours. The identities of the synthesized peptides were confirmed using a Waters/Micromass (Manchester, UK) ZQ ESI-mass spectrometer in positive ionisation mode prior to use.

Experimental Conditions

The effects of four experimental conditions: i) surface material, ii) temperature, iii) humidity, and iv) fluorophore addition, on reagent stability were investigated. For monitoring the effect of surface material, peptide sequences 1-3 were mixed into MHF medium (Vaseline, UK) at a concentration of 2.5:100 mg of peptide:medium. Mixtures containing sequence 1 and 3 were then applied in 40mg deposits onto 10 different surfaces (ceramic, glass, plastic, aluminium foil, wood, paper, denim, polyester, leather, and cotton) cut into 2cm x 2cm squares. The mixture in sequence 2 was applied in the same method to surfaces of wood and paper (as the stability of this sequence on the other 8 surfaces was already proven within the previous proof of concept study). The surfaces were then left exposed to standard laboratory conditions (temperature and humidity: 23±2 °C, 40±5%), constantly monitored using a data logger. Liquid-liquid extractions were then performed to isolate the peptides for ESI-MS analysis at time points of two weeks, four weeks, and six weeks for a total period of six weeks.

For experimental condition studies ii), iii), and iv), the same concentration of peptide-MHF medium was applied to glass slides and exposed to their respective conditions. The effect of temperature was assessed via incubation of slides at both 4°C and 39°C and extracted at time points of six hours, 24 hours, one week, and six weeks. For the effect of humidity, surfaces were left in containers of 70% and 90% (as monitored by a hygrometer) and were once again extracted at the same time points as the temperature study. Humidity containers were prepared by enclosing wet paper towels within plastic boxes.

The effect on fluorophore presence on peptide detection was observed through the addition of Rhodamine-110 (Sigma Aldrich, UK) to peptide-MHF at a concentration of 0.25:100 mg fluorophore:peptide-medium. This mixture was then applied in 40mg deposits on glass slides, which were then exposed to the standard, temperature and humidity conditions previously investigated. Extractions of fluorophore-peptide-MHF reagent were undertaken at time points of three weeks and six weeks for a total period of six weeks.

Taggant Extraction and Analysis

Extraction surfaces were placed into a 30 mL multipurpose container (Greiner Bio-One, UK) before the addition of 1 mL of hexane. Samples were vortexed for 1 minute and the surfaces were removed using sterile forceps prior to the addition of 0.5 mL of distilled water. The samples were vortexed again

and allowed to settle before the aqueous phase was removed into a fresh container via pipette. The original organic phase was then further extracted with an additional 0.5 ml of distilled water before both aqueous layers were pooled together. Counter extraction against the pooled aqueous layers was next performed with 1mL of hexane. The final aqueous layer was then isolated into an Eppendorf tube before the addition of 0.1% v/v Formic Acid to enhance ionisation. ESI-MS analysis of the samples was conducted using a Waters ZQ micromass 2000 mass spectrometer in positive ionization.

Casework Application

A thin layer of peptide reagent (containing sequence 2) was spread across a 25 x 25 cm glass tile to mimic a real-world application to a known surface. A transparent latex glove worn over the hand (with the glove pre-weighted) was then used to contact the surface at a standardised force of ~20 N (monitored by analytical balance). Transferred reagent was visualised using a Crime-lite handheld light source (Foster and Freeman, UK) at an excitation wavelength of 460-510 nm and a supplied 495 nm viewing filter. The glove was then reweighed to assess the amount of taggant transferred upon physical contact.

Depletion Series

To establish the overall sensitivity of the reagent and determine its persistence after multiple contact transfers, a depletion series was prepared. A thin layer of peptide reagent (containing sequence 2) was once again spread over a glass tile before being contacted by a single gloved finger at a standardised force of ~10 N. A series of contacts after the initial reagent transfer were then performed by successively touching either 1, 2, 3, 4 or 5 clean glass slides with the glove. A sterile cotton swab of the entire surface of the finger was used to collect any taggant remaining at the end of each depletion series. Peptide sequence 2 was lastly extracted from all of the swabs and analysed using the methods previously described.

RESULTS AND DISCUSSION

Peptide Synthesis

Three different peptides were used to demonstrate the flexibility of the reagents coding system. These sequences were chosen due to their hydrophilicity, allowing them to be easily isolated from the non-polar MHF medium by simple liquid-liquid extraction. The properties of each peptide, including hydrophilicity, were derived from GenScript's peptide property calculator (Table 1).

The sequences were also ensured to be non-naturally occurring using UniProt's BLAST function, thereby reducing the possibility of generating false positive results from ubiquitous environmental peptides.

Developing a coding system that is able to remain stable and recoverable over extended periods of time is a crucial aspect of taggant design. We therefore decided to explore the effect of a number of modifications on our peptide sequences, with the ideal aim of maximising code detection post taggant recovery. N-terminal acetylation was carried out on all peptides to increase resistance to degradation by aminopeptidase activity¹⁰.

Further protection against enzymatic destruction was conferred by the C-terminal amidation¹¹ of sequence 1 (Ac-KQQSP-NH₂) and the introduction of D-amino acids in peptide sequence 3 (Ac-hsskl-COOH). ESI-MS analysis was used to confirm the identity of the synthesized products, which all exhibited correct masses (See Figure S1).

Table 1. Summary of peptide characteristics obtained from GenScript's peptide property calculator

Peptide Sequence 1: Ac-KQQSP-NH₂ (L-amino acids)	
GenScript PPC	MW= 627.69, Attribute= Basic (Hydrophilic)
Peptide Sequence 2: Ac-HSSKL-COOH (L-amino acids)	
GenScript PPC	MW= 612.68, Attribute= Basic (Hydrophilic)
Peptide Sequence 3: Ac-hsskl-COOH (D-amino acids)	
GenScript PPC	MW= 612.68, Attribute= Basic (Hydrophilic)

Experimental Conditions

With popular uses in marking vehicles, objects, fabrics and premises, the nature of the surface on which a taggant may be applied can be highly variable. It is consequently crucial that newly developed taggants demonstrate sufficient robustness to be detected and identified over extended periods of time, without being affected by the material on which they are deposited. The stability of all three peptide-MHF formulations was therefore monitored by their application on 10 different surfaces routinely encountered during forensic casework. All taggants were then left in standard laboratory conditions (temperature and humidity: 23±2 °C, 40±5%) before being extracted at several time points over a period of six weeks. Figure 1 shows the mass spectra of analysed reagent samples containing peptide sequence 1, 2 and 3 from paper at the 6-week time point. The same results were achieved across all peptide, surface and time combinations, with no observable reagent degradation (See Figure S2-S4).

Figure 1. ESI-MS spectra of a) peptide sequence 1: Ac-KQQSP-NH₂, (theoretical [M+H]⁺ m/z ratio = 628.34) b) peptide sequence 2: Ac-HSSKL-COOH (theoretical [M+H]⁺ m/z ratio = 613.33) and c) peptide sequence 3: Ac-hsskl-COOH (theoretical [M+H]⁺ m/z ratio = 613.33) reagent samples extracted six weeks after application to paper. Similar results were seen on all other surfaces.

Another facet of forensic taggant application that is highly variable is the environmental conditions to which a reagent may be exposed.¹² By marking items both outside and in sheltered locations, taggants are often subjected to a wide range of temperatures and humidities. However, the effect of such conditions on the detection and identification a taggant should ideally remain negligible. To establish the environmental resistance of our peptide coding reagent, formulations containing sequences 1-3 were deposited on glass slides and incubated over six weeks in temperatures of 4°C and 39°C or in humidity's of 70% and 90%. Extraction and analysis of the reagents at various time points demonstrated that all peptides remained detectable despite incubation conditions, with no differentiation in the achieved mass spectra being displayed between samples (See Figure S5-S6).

The foremost purpose of Rhodamine-110 addition to the peptide-MHF formulation is to allow taggants to be detected via fluorescence emission after transfer. This fluorescence not only quickly establishes evidence of contact between an object and a suspect but may also be used for a more targeted swabbing by indicating where identifying peptides are likely to be present. Moreover, as water-soluble Rhodamine-110 is co-extracted with peptides into the same aqueous phase, a dual detection of the reagent is provided by the presence of both fluorophore and peptide ion peaks in the ESI-MS spectrum.

Figure 2. ESI-MS spectrum of Sequence 1: Ac-KQQSP-NH₂ (theoretical [M+H]⁺ m/z ratio = 628.34, theoretical [M+Na]⁺ m/z ratio = 650.32) and Rhodamine-110 (theoretical [M+H]⁺ m/z ratio = 331.11) extracted from full reagent samples applied to glass slides and incubated for six weeks in standard laboratory conditions.

To establish this dual-detectability, three final fluorophore-peptide-MHF formulations for each of the peptide sequences utilised were prepared and deposited on glass slides in the same manner previously described. Slides were exposed over six weeks to the standard laboratory, temperature and humidity conditions already explored and were once again extracted and analysed at a number of time points. Figure 2 displays both fluorophore and peptide components detected by ESI-MS after extraction of final reagent (containing peptide sequence 1) after six weeks of standard laboratory exposure. In all cases, similar results were achieved regardless of peptide or environmental condition, with no observable fluorophore or peptide degradation (See Figure S7-S9).

Casework Application and Depletion Series

To successfully operate in the field, touch contacts must result in enough fluorophore and peptide transfer to ensure taggant visualisation and identification respectively. Demonstra-

tion of the reagents practical use in a real world scenario was therefore achieved

Figure 3. Peptide coding reagent successfully transferred upon physical contact. Left: prior to visualisation. Right: fluorescence emission upon excitation.

by applying a thin layer of fluorophore-peptide-MHF formulation over a large glass tile, which was subsequently contacted by a transparent glove worn over the hand. As expected, the reagent remained imperceptible under natural light but was easily viewed upon the hand after illumination with a portable light source (Figure 3). Approximately 0.44 mg of taggant was transferred to each cm² of the glove's surface.

Whilst this rate of transfer may be considered ideal after an initial contact, the amount of taggant recovered in a practical setting may actually be far less, depending upon the subsequent activity of the offender. For example, it is possible that the reagent may be removed if a suspect contacts other surfaces after touching a marked object, thereby diminishing the amount of peptide available for analysis. Designing a taggant to persist on an individual even after multiple contacts is therefore important. A depletion series was created to measure the overall retention of our reagent by contacting the tile previously prepared with a gloved finger. Taggant was then collected and recovered from the entire surface of the finger by direct swabbing and liquid-liquid extraction respectively. This process was then repeated but with additional secondary contacts on clean glass slides (increasing from 1 to 5 contacts) prior to reagent recovery. ESI-MS analysis successfully detected the presence of both peptide and fluorophore ion peaks within all samples, establishing the excellent persistence of our reagent (See Figure S10).

CONCLUSIONS

The ability of a novel 'peptide coding' taggant to monitor contact transfer during criminal activity has been successfully established. The reagent is invisible to the naked eye but highly fluorescent when excited by an appropriate light source. If recovered from an individual, this reagent has the power to physically link a person to a specific object or offence through uniquely identifying amino acid sequences. The highly transferability of the employed medium allows these peptides to persist on a person even after five additional contacts with other surfaces.

Remaining detectable and recoverable over a period of six weeks, our developed peptide taggant has proven to be successfully stable and resistant to all variable experimental conditions. A greater degree of confidence can therefore be given in the taggants efficacy to operate over extended periods of time on different surfaces and in a range of environments.

This reagent promises significant advantages over currently available forensic taggants; liquid-liquid extraction allows coding sequences to be rapidly isolated with little expense. Taggant analysis via ESI-MS is also significantly less time consuming than sequencing techniques used in DNA-based taggants and may be completed within 1 hour of reagent recovery. Continuing advances in portable mass spectrometry technology are likely to result in this time being further reduced, by allowing analysis to be performed at the point of taggant discovery.

Whilst not explored further within this study, this taggant was designed with a high intrinsic flexibility so that it may be easily adapted for application in a variety of crime reduction strategies. This is likely to centre on the substitution of the reagents individual components to suit a specific purpose. For example, the replacement of Rhodamine-110 with a near-infrared fluorophores may provide enhanced contrast against certain coloured or auto-fluorescent surfaces¹³. The discriminatory power of the taggants code may also be further increased by the use amino acids that feature unnatural modifications. Whilst this reagent was developed for observing physical contact transfer during criminal activity, many taggants are designed to remain immobile upon the object they mark. Our peptide coding system may also be utilised in such an application by a simple change of dispersal media.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

ESI-MS spectra (Figure S1) of synthesized peptides and extracted reagent samples (Figures S2-S10) are included. (PDF)

Authors are required to submit a graphic entry for the Table of Contents (TOC) that, in conjunction with the manuscript title, should give the reader a representative idea of one of the following: A key structure, reaction, equation, concept, or theorem, etc., that is discussed in the manuscript. Consult the journal's Instructions for Authors for TOC graphic specifications.

AUTHOR INFORMATION

Corresponding Author

* Email: nunzianda.frascione@kcl.ac.uk Tel: +442078484978

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