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Practical fluorescence detection of acrolein in human plasma *via* a two-step tethering approach

A simple and high-throughput method to measure the concentration of acrolein, a cytotoxic α,β-unsaturated aldehyde and disease biomarker, in human plasma was developed. The method relies on a novel tethering approach using hydrazine microbeads and a thiol-containing fluorophore.

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Practical fluorescence detection of acrolein in human plasma *via* a two-step tethering approach†

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Acrolein, a cytotoxic α,β-unsaturated aldehyde and disease biomarker, was determined in plasma by means of a novel tethering strategy using Michael addition of the compound to a fluorescent dye, followed by immobilization of the product on microbeads *via* the aldehyde moiety. Elevation of blood acrolein was detected in mice treated with an anticancer agent cyclophosphamide, which releases acrolein upon activation. This method should be suitable for high-throughput diagnostic and clinical application.

Acrolein (CH_2CHCHO) is a strong electrophile that disrupts important cellular functions by covalently modifying biomolecules, including proteins and DNA.[1] Formerly, it was thought that acrolein is generated from unsaturated lipids and reactive oxygen species (ROS),[2] but recent reports indicate that acrolein is mainly produced from polyamines, such as spermine, by polyamine oxidases (PAOs).[3] Acrolein and its protein conjugate are of interest as disease markers. For example, they were reported to be increased in plasma of patients with chronic renal failure[4] or brain infarction.[3b,5] Therefore, quantification of acrolein in biological samples such as plasma or urine is potentially of diagnostic value. However, conventional methods for detection of acrolein are not suitable for high-throughput assay, and hence are not applicable for diagnosis. A practical detection method for pathological concentrations of free acrolein (sub to low micromolar range)[3a,4] is expected to find many applications in the medical and biological fields.

The most widely used method for detecting free acrolein in biological samples is fluorescence analysis based on the Skraup reaction with 3-aminophenol.[6] In this method, the plasma of patients is reacted with 3-aminophenol under acidic conditions, and the fluorescent product, 7-hydroxyquinoline, is quantified. In most cases,[6a,b] detection after HPLC separation is necessary to obtain a sufficient S/N ratio, because (i) the background fluorescence of plasma is high upon excitation at 350–400 nm (the excitation wavelength of 7-hydroxyquinoline), and (ii) the fluorescence quantum yield of 7-hydroxyquinoline is low. However, HPLC is unsuitable for high-throughput assay of multiple samples. In addition, the reaction conditions of the conventional assay are quite severe (1 N HCl aq., reflux), which is also inconvenient for practical measurement on site. Acrolein–protein or –DNA adducts can be detected using monoclonal antibodies,[2a,7] but the method is costly and requires complicated procedures. Moreover, there is a delay of several hours between the production of acrolein and the formation of acrolein adducts. Therefore, in order to overcome the limitations of current methods, we have developed a novel, inexpensive, and practical fluorescence-based method, using a two-step tethering strategy, for detection of acrolein under mild conditions without need for HPLC.

For fluorescence detection of acrolein at the low concentrations that exist in biological samples, including plasma and urine, we considered that it would be essential to remove background signals by isolation of the target compound. As the use of HPLC is undesirable for the reasons mentioned above, we considered that a two-step tethering procedure to a solid phase, taking advantage of the characteristic α,β-unsaturated carbonyl reactivity, would be an effective alternative. Our design strategy is summarized in Fig. 1.

First, a fluorescent compound with a thiol functional group (I) is added to acrolein to yield the Michael addition product (II). Second, the mixture is incubated with plastic microbeads bearing hydrazine functional groups on their surface, resulting in concentration-dependent covalent attachment of the fluorophore-bearing addition product to the microbeads (III). Unbound dye and biological components (proteins, sugars, nucleic acids, *etc.*) can then be readily removed simply by washing the microbeads, as already well established.[8]

We selected tetramethylcarboxyrhodamine (TAMRA) as the fluorophore because it emits strong red fluorescence and has

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† Electronic supplementary information (ESI) available: Table S1, Fig. S1, and experimental section. See DOI: 10.1039/c4cc02578d

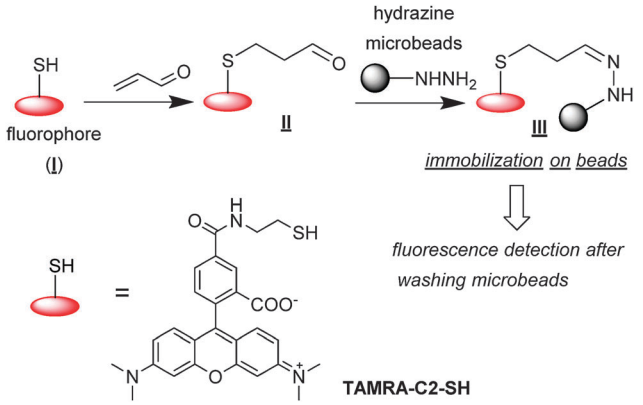

Fig. 1 Schematic representation of the strategy for detection of acrolein in biological samples and the structure of the thiol-containing fluorescent compound used in this work, TAMRA-C2-SH.

high solubility in water, and synthesized TAMRA-C2-SH (Fig. 1). The synthetic procedures and photophysical properties of TAMRA-C2-SH are described in the ESI.† As microbeads, we selected a PEG-attached resin (TentaGel) with hydrazine functional groups, because it has excellent swelling properties in aqueous solution. Then we optimized the reaction conditions of acrolein, TAMRA-C2-SH, and microbeads. The optimum conditions were as follows: TAMRA-C2-SH and acrolein in sodium phosphate buffer (pH 7.4) were first incubated at 40 °C for 1 h, then the pH was adjusted to 3.5 with sodium phosphate buffer (pH 1.4). Second, TentaGel-NH-NH_2 microbeads were added to the mixture, followed by incubation at 40 °C for 3 h and brief washing with DMF and sodium phosphate buffer (pH 7.4). Fig. 2 shows confocal fluorescence microscopic images of microbeads after the two-step tethering process. The beads incubated in the acrolein-containing solution emitted strong fluorescence, while those incubated in the acrolein-free solution emitted almost no fluorescence. These results showed that our tethering strategy, in which acrolein serves as a linker between the microbeads and TAMRA-C2-SH, is effective, and that non-specific adsorption of the fluorophore on the beads is negligible. Therefore, we next investigated the selectivity of our method. As shown in Fig. 3, the method was very selective for α,β-unsaturated

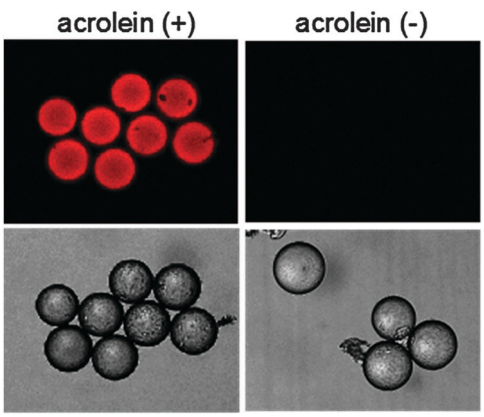

Fig. 2 Confocal fluorescence microscopic images of microbeads incubated with acrolein-containing solution (acrolein: 10 μM, left) and acrolein-free solution (right). Upper panel; fluorescence images, lower panel; bright-field images.

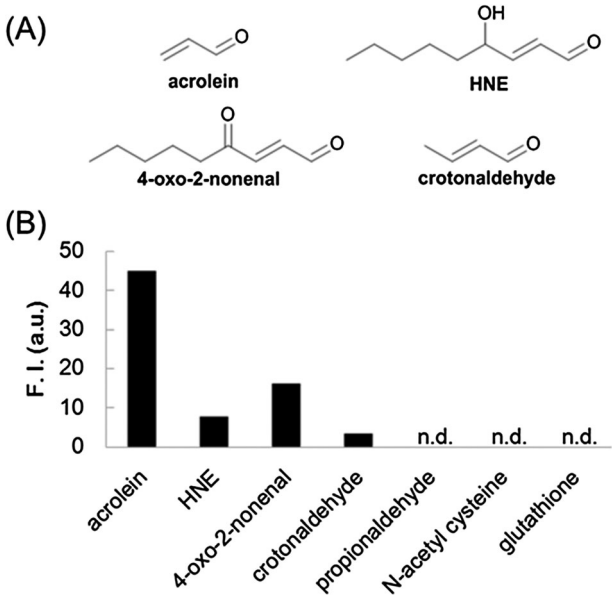

Fig. 3 Fluorescence assays of various α,β-unsaturated carbonyl compounds, propionaldehyde, *N*-acetylcysteine and glutathione (5 μM each). (A) Structures of the analytes used. (B) Fluorescence intensity (ex./em. = 554/570–700 nm) of the microbeads, which was recorded using confocal fluorescence microscopy; n.d.: not detected (F.I. < 0.1).

carbonyl compounds, and the highest reactivity was observed for acrolein, presumably due to its small size. We also carried out HPLC analysis to confirm that the reaction proceeded as we expected (see the ESI†). Since the biological concentration of acrolein is higher than those of other α,β-unsaturated carbonyl compounds such as 4-hydroxynonenal (HNE),[5] we consider that our method has sufficient specificity for acrolein for practical purposes in a biological context.

We then applied this detection method to human plasma. Plasma itself has absorbance in the UV-visible region, and emits fluorescence in the range of 300–500 nm. Therefore, the conventional 7-hydroxyquinoline method gives poor results in plasma. However, with our method, the background signal is easily eliminated by washing the microbeads. As can be seen in Fig. 4A, as little as 1 μM acrolein could be reliably detected with our method, and the fluorescence increased linearly as a function of added acrolein concentration in plasma.[9] In contrast, 1 μM acrolein could not be detected after derivatization with 3-aminophenol

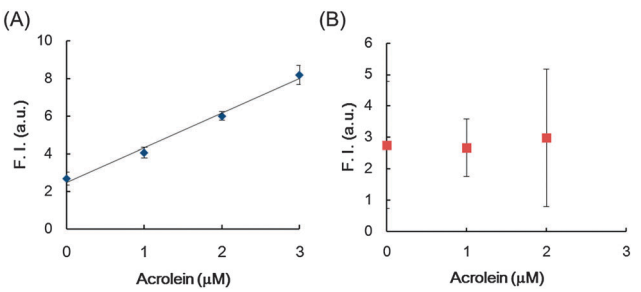

Fig. 4 (A) Fluorescence assays of acrolein (0, 1, 2, 3 μM) in plasma with TAMRA-C2-SH and TentaGel-NH-NH_2 microbeads. Fluorescence intensity (ex./em. = 554/575 nm) was recorded after washing the microbeads. (B) Fluorescence assays in plasma using the conventional 7-hydroxyquinoline method. Data are shown as mean ± SD (n = 3).

in the absence of HPLC separation (Fig. 4B). With the conventional method, quantitative assays in this concentration range are not possible because of the intrinsic background fluorescence of plasma. The calculated detection limit of our method was 0.54 μM, which is one order of magnitude smaller than that of the conventional method (5.4 μM) without HPLC separation.

Finally, we applied the method for detection of acrolein in plasma of mice treated with cyclophosphamide (CPA). CPA and its analog ifosfamide are well-known anticancer agents.[10] However, these drugs are metabolically activated by the liver P450 system, and then decomposed into phosphoramide mustards and acrolein.[10a,e] Although a good method to quantify activated CPA has been reported with the aid of HPLC,[11] it would be preferable to monitor the total acrolein concentration in the blood of CPA-treated patients because acrolein is likely to be responsible for the adverse effect of hemorrhagic cystitis observed in some patients.[10b]

Therefore, to demonstrate that our method has practical value and is sufficiently sensitive for clinical use, we used it to determine the acrolein concentration in plasma of CPA-treated and non-treated mice. CPA (0.4 mg per body) in saline was injected intravenously, and then 30 min later, the mice were sacrificed and the plasma was collected. As shown in Fig. 5, the acrolein concentration in plasma was markedly increased in CPA-treated mice. The results clearly demonstrate that our method can detect acrolein generated *in vivo*.

In conclusion, we have developed a novel, inexpensive, and practical fluorescence-based method for detecting acrolein in human plasma. Our method relies on the characteristic reactivity of α,β-unsaturated aldehydes to selectively tether a fluorophore (TAMRA-C2-SH) to a solid phase (TentaGel microbeads), using the analyte as a linker. It enables sensitive (submicromolar) and practical detection of acrolein in plasma without the need for HPLC separation. We confirmed its utility by detecting the elevation of acrolein in plasma of CPA-treated mice, as a model of a serious side effect in human cancer patients.

Compared with most fluorescent probes for specific biomolecules,[12] which are elaborately designed conjugates of fluorophore and receptor/substrate moieties, the detection strategy used in this work is quite different, in that reaction with the target does not alter the fluorescence properties of the probe itself. Nevertheless, high selectivity and sensitivity are obtained because unsaturated aldehyde works as a linker to tether the fluorophore on microbeads. Although this is not the first application of a tethering strategy, only very limited examples have so far been reported for a few other biological molecules.[8] Here, we used plastic microbeads as a solid phase because of their ready commercial availability, but it should be straightforward to employ other materials, such as multi-well plates or glass slides functionalized with hydrazine for convenient high-throughput application. A potential drawback of this strategy may be the lack of strict discrimination of acrolein from other α,β-unsaturated carbonyl compounds, but we believe this is not a bar to practical application for diagnostic and clinical purposes, as discussed above. Some of the present authors are currently validating this method for biological and clinical use,[13] with the expectation that it will improve patient safety. It should also be useful in studies of the pathological mechanisms of acrolein.

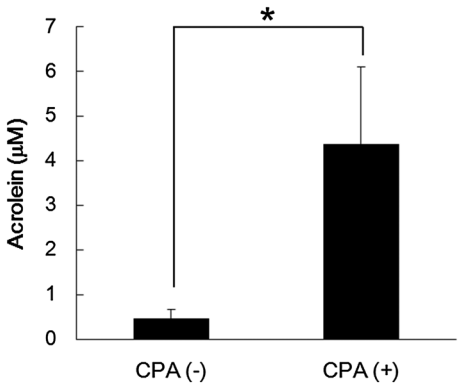

Fig. 5 Determination of acrolein in plasma of CPA-treated and non-treated mice. Data are shown as mean ± SD (n = 3). * indicates $p < 0.05$ (Student's t-test).

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