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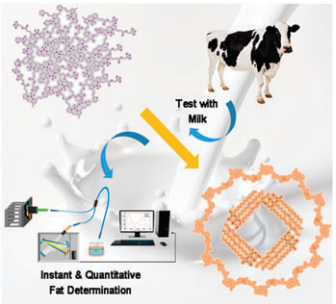

Milk quality control: instant and quantitative milk fat determination with a BODIPY sensor-based fluorescence detector Q1 Q2

Wang Xu, Jiaojiao Bai, Juanjuan Peng,
Animesh Samanta, Divyanshu and Young-Tae Chang* Q3

The first fluorescent sensor for milk fat was developed. Q4

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Milk quality control: instant and quantitative milk fat determination with a BODIPY sensor-based fluorescence detector†

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The first fluorescent sensor for milk fat was developed. It exhibited a magnificent, yet selective turn-on feature towards fat molecules in a complicated milk matrix by a disaggregation-induced emission mechanism. Further construction of a handy fluorescence milk fat detector provided a convenient rapid tool to measure the fat amount quantitatively. This discovery may help enhance the milk quality control process.

Milk, whose nutritional value long been recognized by our ancestors thousands of years ago, is consumed regularly by over 6 billion people throughout the world. Today, the whole dairy industry, including production, procession and delivery, has become an astonishingly huge multi-billion dollar industry.[1] Fat, the major energy contributor in milk, has attracted thorough but not complete investigations.[2,3] The milk fat amount is recognized as an indicator of milk quality; hence, it is directly correlated with nutritional and marketing value of milk. Therefore, a rapid and sensitive fat detector is in high demand in the milk industry.

The traditional milk fat detection method (Gerber method) is comprised of many procedures, including destabilization of fat molecules (triglycerides) by mixing milk with sulphuric acid, separation of the fatty acids from aqueous phase by centrifugation and volume-based measurement of the upper-layer hydrophobic fatty acids using a butyrometer.[4,5] The complicated handling, inevitable usage of toxic corrosive chemicals and high cost make it unfavourable for non-trained hands. Instruments utilizing the infrared absorbance of specific bond vibrations are available; however, their cost and sophisticated configuration prevents their prevalence in resource limited regions.[6,7] Fluorescence spectroscopy, owing to its outstanding sensitivity and selectivity, as well as its straightforward signal output and operation, has attracted significant research interest.[8,9] Our group has developed diversity-oriented fluorescence libraries (DOFL), which have proven their versatile applications in sensor development.[10] In this report, by screening our libraries towards milk samples, we successfully identified one BODIPY compound that shows remarkable fluorescence signal increments with increasing concentrations of fat in milk. Moreover, we demonstrated that this sensor could instantly and quantitatively determine fat amount in the given samples, regardless of the brands of milk tested. We further developed this sensor into a fat detection device, utilizing inexpensive and readily acquirable materials. We hold faith that this fat detector could significantly enhance the milk quality control process, especially in resource-limited regions, as well as greatly influencing the multi-billion milk industry.

Fig. 1 gives the structure and photophysical properties of the milk fat sensor. Firstly, more than 10 000 fluorescent dyes were

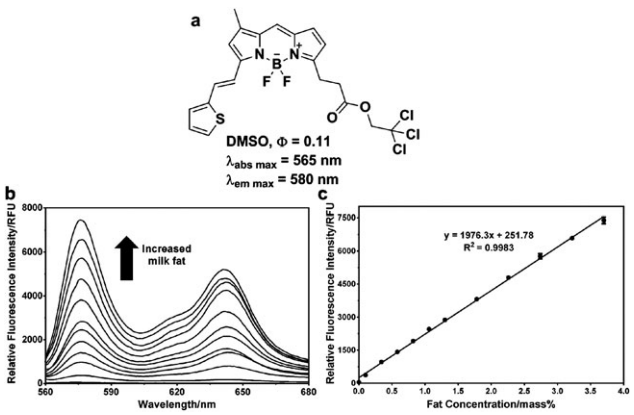

Fig. 1 Structure and photophysical properties of MO. (a) Structure and spectral information of MO. (b) Fluorescence spectra of MO–fat interaction. Milk fat concentration increases from 0.1% to 3.7% by mass and MO concentration is 10 μM. Excitation is 530 nm. (c) Dose-dependent linear response graph of fluorescence intensity towards fat concentration.

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designed, synthesized and purified.[11] The huge structural diversity allows better chance of sensor discovery, especially to analytes that are almost impossible to design binding moieties for. For example, due to the complicated matrix in milk, such as proteins, carbohydrates and ions, it is impossible to design a binding moiety for fat, majority of which is triglycerides, without being disturbed by any other existing substances.[12] Hence, the screening of DOFL becomes the best solution for this problem. To facilitate the selection process, given the huge number of dyes available (>10 000), we constructed one imaging black box.[13] Simply irradiating the fluorescent dyes with a light source and taking pictures before and after the addition of milk samples using a normal camera, we can acquire fluorescence intensity/wavelength changes induced by milk (Fig. S1 and S2, ESI†). To maximize the chance of sensor discovery, we carefully measured the auto-fluorescence background of milk itself and serially diluted it until the background was fully quenched such that any change in the fluorescence signals would be derived from the dye itself (Fig. S3, ESI†). The final milk sample has been 100-time diluted, and it does not exhibit any detectable background. With the help of this hyper-throughput imaging system, we were able to rapidly screen thousands of fluorescent dyes in a single day, in the format of 96-well plates.

After collecting the candidates by analyzing the pictures and selecting any compound that exhibits dramatic signal variation, we proceeded to the confirmation step with a professional spectrometer and measured the dose-dependent spectral change in milk fat towards the selected fluorescent dyes (Fig. S1b and S2c, ESI†). One BODIPY dye, which exhibits highest response towards milk fat, was finally identified and named as milk orange (MO) (Fig. 1a, Fig. S4 and Scheme S1, ESI†). Further analysis of the MO–milk response spectra revealed that skim milk shows a very dim signal with MO, which is almost invisible through the naked eye. With the increment of milk fat, the mixture of two emission peaks (580 nm and 640 nm) gives orange fluorescence (Fig. S5a, ESI†). The 580 nm emission was selected to afford a linearity check due to its higher intensity and better linearity. The emission signals exhibited a strong linear response towards milk fat, with a Pearson coefficient of determination, *i.e.* R^2, of more than 0.99 (Fig. 1b and c). This indicates that MO has great potential to be directly applied to quantitatively measure the fat amount in milk samples.

To understand the sensing mechanism of the MO–milk interaction, we performed both ^{19}F nuclear magnetic resonance (NMR) and dynamic light scattering (DLS) studies. It is known that BODIPY shows an important aggregation feature in aqueous solutions due to its highly hydrophobic nature.[13–15] In an aqueous environment, BODIPY dyes are surrounded by water molecules; hence, they are forced to compile together and create self-quenched, non-fluorescent aggregates. On the other hand, milk fat is primarily composed of triglyceride molecules, which could create a perfect hydrophobic region for BODIPY dyes. It is highly reasonable that MO along in an aqueous solution would not reveal a fluorine signal in NMR spectroscopy due to its aggregated conformation. Thus, the addition of

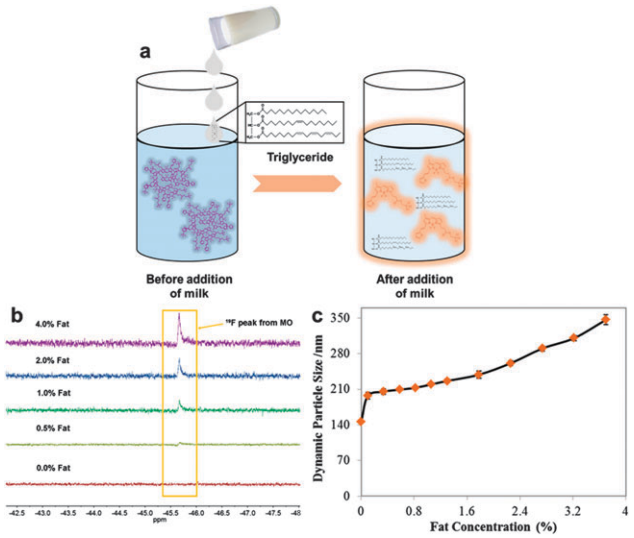

Fig. 2 MO disaggregation-induced emission. (a) Schematic diagram of MO disaggregation while adding milk that contains triglycerides (fat). (b) ^{19}F NMR of MO with various concentrations of extracted triglyceride. (c) Dynamic radius of the MO–milk composites.

fat molecules should visualize this peak (Fig. 2a). Inspired by this possibility, we first performed ^{19}F NMR of MO in an aqueous solution since it is easy to analyze. It is clearly seen that MO has no fluorine peak in D_2O. To minimize the disturbances from the milk matrix, we extracted milk with dichloromethane to afford relatively pure triglyceride molecules.[12] This molecule was added to the MO aqueous solution and its NMR was tested again. We clearly observed a ^{19}F peak at −45.67 ppm. Furthermore, increases in the triglyceride concentration led to a higher peak, which corroborates our hypothesis of disaggregation-induced fluorescence enhancement (Fig. 2b).

To further confirm this phenomenon in a more realistic environment, we explored the MO–milk particle size changes with DLS studies. The MO scaffold is strongly squeezed by water molecules and forms substantial aggregates with an approximate diameter of 140 nm. The addition of milk at the same concentration as in the fluorescence tests provides a hydrophobic environment within the lipid chains of triglycerides, which is remarkably beneficial for MO disaggregation, and thus results in BODIPY fluorescence. With increasing amount of milk fat, the dynamic radius increased to 350 nm at 3.7% fat (Fig. 2c). Furthermore, MO structure contains methoxy and fluorine groups, which can form hydrogen bonds with the triglycerides in milk fat. The specific interactions between MO and milk fat further enhance the disaggregation of MO molecules.[16]

Before applying MO to real-life fat measurement, we need to confirm that it only responds to fat, and not to any other substances in milk. Moreover, MO should not have any prejudice against milk from different origins. To fully explore its applicability, we tested MO in diversified milk samples, which are readily available products collected in a local market (Fig. 3). They were consistently homogenized and labelled with exact amounts of all ingredients. In each brand, we secured as

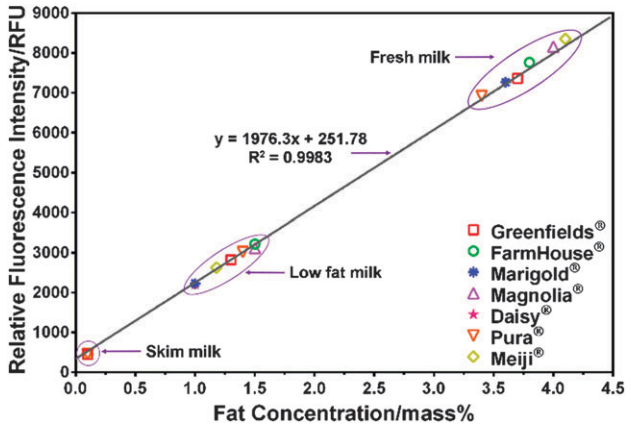

Fig. 3 Selectivity test of MO. A straight line is derived from one type of milk (Greenfields®). All other data sets are plotted on the graph based on the respective fat concentration and fluorescence intensity of each type of milk.

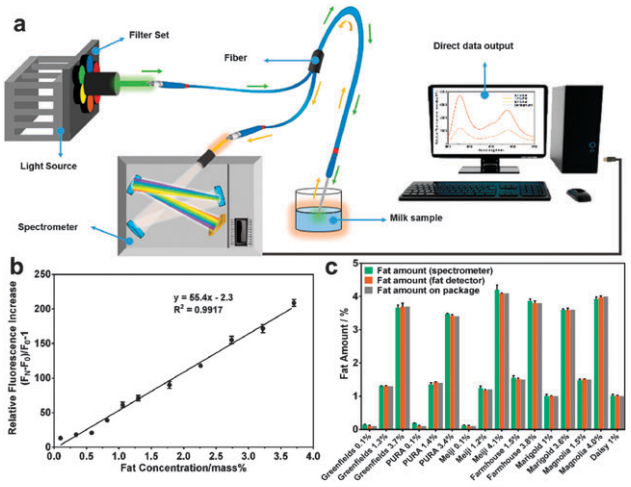

Fig. 4 Construction of a rapid quantitative milk fat detector. (a) Scheme of each component of the milk fat detector. (b) Dose-dependent linear response graph of fluorescence intensity towards the fat concentration measured using the milk fat detector. (c) Comparison chart of fat amounts acquired from a spectrometer and fat detector. The green bar indicates the spectrometer data, orange bar is fat detector data, and the grey bar is the fat amount labelled on the package.

many types of milk as possible after ensuring consistent milk processing procedures and presence of largely similar ingredients. By measuring their fluorescence intensity and correlating with the dose-dependent linear response curve acquired, we successfully achieved quantitative measurements of fat from all brands of milk with an error less than 1.5% (Table S1, ESI†). Fig. 3 shows the plot of all types of milk according to their respective fat concentrations and measured relative fluorescence intensities on the linear response graph derived from one type of milk (Greenfields®). All spots lie perfectly on a straight line, indicating that MO can be applied universally to various types of milk, and their origins (Australia, New Zealand, Thailand) do not affect the fat measurement.

Furthermore, Table S1 (ESI†) clearly shows that among all 16 types of milk from 7 brands, their protein amounts ranged from 3.0% to 5.0%, while the carbohydrate level has an even higher range of 4.8% to 7.0%. Nevertheless, none of these two species posed any disturbance to the fluorescence measurement of fat amount. A parallel comparison among the skim milk, low fat milk and fresh milk could provide more information. For instance, in skim milk, although all of samples contained 0.1% fat, the protein concentrations varied from 3.2% to 4.0%, while their carbohydrate concentrations varied from 4.2% to 6.9%. This dramatic difference within other ingredients does not affect the fat measurement. Similarly, the protein amount in low fat milk varied from 3.2% to 5.0%, while the carbohydrate amount varied from 3.9% to 7.0%. Among the fresh milk samples, the protein amount varied from 3.2% to 4.0%, while carbohydrate varied from 3.9% to 5.0%. We can thus conclude that neither the protein nor carbohydrate would affect the fat measurement. This demonstrates the remarkable selectivity and versatility of MO towards milk fat (Fig. S5b and Tables S2–S9, ESI†).

Prior to MO, no fluorescence milk fat sensor or any practically applicable and customer-friendly fluorescence milk fat detector has been reported. In order to fully maximize the potential of MO, we incorporated it with an easily achievable and handy fluorescence detector. In brief, the detector is composed of one light source, one light passing channel and one grating fluorescence detector connected to a computer (Fig. 4a). For experimental purposes, we adopted one white LED light source with a green (520 ± 5 nm) filter. Green excitation light passes through the optical fibre that both emits light and receives it at the same probe tip. The probe tip is immersed into the milk sample with its green light directly irradiating the sample while receiving the fluorescence signals. The optical fibre thus transfers the fluorescence signals to a grating fluorescence detector that instantly separates the incoming light into the whole spectrum (300–1100 nm) and transmits the detected photon numbers to a computer, which draws the corresponding spectra (Fig. S6, ESI†). Fig. 4b shows the linear response derived from the spectra of the fat detector. Its R^2 value was comparable to that acquired from a complicated spectrometer, indicating that the approach to simplify the fat detection process and cost does not affect its sensitivity or accuracy. Moreover, Fig. 4c shows a comparison of fat amounts from all 16 types of milk measured by either the spectrometer or fat detector. It is clear that both values are highly consistent with the fat amounts labelled on the package.

In summary, using a fluorescence image-based high-throughput screening approach, we identified MO as a novel and unprecedented milk fat sensor, which exhibits a remarkable fluorescence turn-on feature towards the fat content. Its fluorescence intensity followed a perfect linear dose-dependent feature with regards to the fat amount, which greatly facilitates milk fat quantitation. The sensing process is based on the disaggregation of MO aggregates in aqueous solution, which results in significant optical changes. Furthermore, the selectivity and versatility of MO has proven its great potential for wide applications to milk from various origins at various quality. The combination of MO with a simple fluorescence detector has opened a new window to practically applying various fluorescence sensors to real life testing. In total, MO

provides a convenient tool for rapid on-site milk fat detection, which could greatly help improve the current milk quality control process.

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