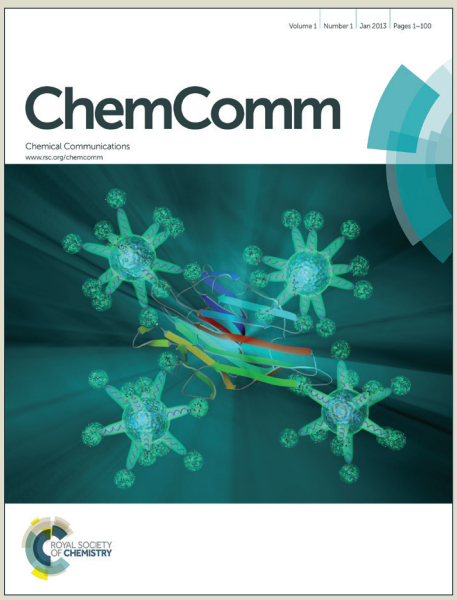
# ChemComm

Accepted Manuscript





ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Chemical Communications RSCPublishing

COMMUNICATION

# Milk Quality Control: Instant and Quantitative Milk Fat Determination with a BODIPY Sensor-based Fluorescence Detector

Cite this: DOI: 10.1039/x0xx00000x

Wang Xu, [a, b] Jiaojiao Bai, [a] Juanjuan Peng, [a] Animesh Samanta, [a] Divyanshu [c] and Young-Tae Chang *, [a]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

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

Milk, with its 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 field, including production, procession and delivery, has become an astonishingly huge industry that occupies billions of dollars annually.[1] Fat, as the major energy contributor in milk, has attracted thorough but not complete investigations.[2, 3] Milk fat amount is recognized as an indicator of milk quality, and hence directly correlated with its nutritional and marketing value. Therefore a rapid and sensitive fat detector is highly demanded in milk industry.

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 subsequently, volume based measurement of upper-layer hydrophobic fatty acids using a butyrometer.[4, 5] The complicated handling, inevitable usage of toxic corrosive chemicals and expensive cost make it unfavourable for non-trained hands. Instruments utilizing infrared absorbance of specific bond vibrations are available, however their costs and sophisticated configuration prevents their prevalence in resource limited regions.[6, 7] Fluorescence spectroscopy, due to its outstanding sensitivity and selectivity, as well as its straightforward signal output and operation, has attracted significant research interests.[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 have demonstrated that this sensor could instantly and quantitatively determine fat amount in given samples, regardless of the brands of milk tested. We further developed this sensor into a fat detection device, utilizing cheap and readily acquirable materials. We hold faith that this fat detector could significantly enhance milk quality control process especially in resource-limited regions, as well as greatly influencing the multi-billion milk industry.

Figure 1 gives the structure and photophysical properties of milk fat sensor. Firstly, more than 10,000 fluorescent dyes were 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 screening of DOFL becomes the best solution for this problem. To facilitate 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 addition of milk samples using a normal camera, we can acquire fluorescence intensity/wavelength changes induced by milk (Figure S1-S2). To maximize the chance of sensor discovery, we carefully measured the auto-fluorescence background of milk itself and serially diluted it until the background is fully quenched, so that any change of fluorescence signals will be derived from the dye itself (Figure S3). The final milk sample has been 100-time diluted that does not exhibit any detectable background. With the help of this hyper-throughput imaging system, we are able to rapidly screen thousands of fluorescent dyes within one day, in the format of 96-well plates.

After collecting 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 of milk fat towards the selected fluorescent dyes (Figure S1b and Figure S2c). One BODIPY dye which exhibits highest response towards milk fat was finally identified and named as Milk Orange (MO) (Figure 1a and Figure S4, Scheme S1). Further analysis of the MO-milk response spectra reveals that skim milk displays very dim signal with MO, which is almost invisible through naked eyes; with increment of milk fat, mixture of two emission peaks (580 nm and 640 nm) gives an orange fluorescence (Figure S5a). 580 nm

emission was selected to afford linearity check due to its higher intensity and better linearity. The emission signals exhibit highly linear response towards milk fat, with the Pearson Coefficient of Determination $R^2$ more than 0.99 (Figure 1b and 1c). It indicates that MO has great potential to be directly applied to quantitatively measure fat amount in milk samples.

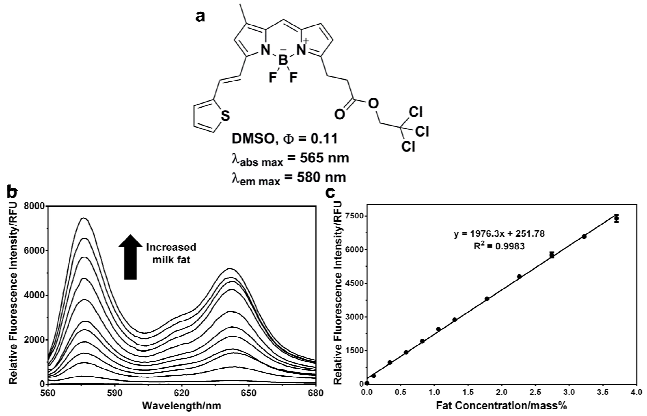



Figure 1. Structure and photophysical properties of MO. (a) Structure and spectral information of MO. (b) Fluorescence spectra of MO-fat interaction. Milk fat concentration increases from 0.1% to 3.7% by mass and MO concentration is 10 μM. Excitation is 530 nm. (c) Dose-dependent linear response graph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

To understand the sensing mechanism of MO-milk interaction, we performed both 19F nuclear magnetic resonance (NMR) and dynamic light scattering (DLS) studies. It is known that BODIPY displays an important aggregation feature in aqueous solutions due to its highly hydrophobic nature.[13-15] In aqueous environment, BODIPY dyes are surrounded by water molecules and hence 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 perfect hydrophobic region for BODIPY dyes. It is highly reasonable that MO along in aqueous solution would not reveal fluorine signal in NMR spectroscopy due to its aggregated conformation. Thus addition of fat molecules should visualize this peak (Figure 2a). Inspired by this possibility, we first performed 19F NMR of MO in aqueous solution since it is easy to analyze. It is clearly seen that MO has no fluorine peak in $D_2O$. To minimize disturbances from milk matrix, we extracted milk with dichloromethane to afford relatively pure triglyceride molecules.[12] This molecule was added to MO aqueous solution and its NMR tested again. We clearly observed appearance of 19F peak at -45.67 ppm. Further increase of triglyceride concentration leads to higher peak, which corroborates our hypothesis of disaggregation-induced fluorescence enhancement (Figure 2b).

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

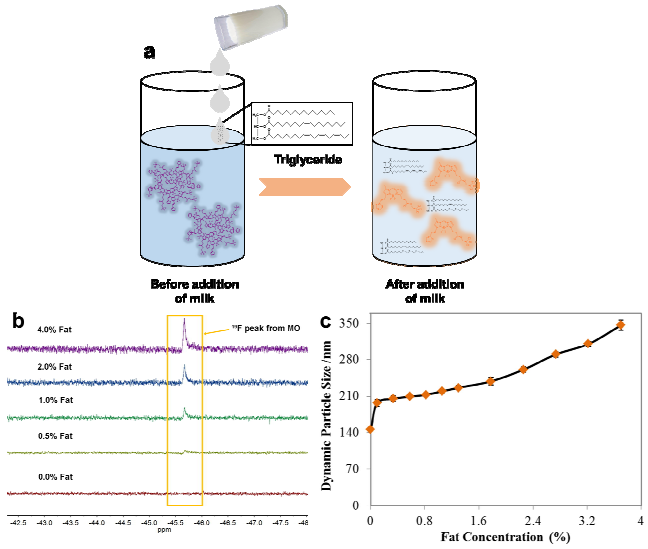



Figure 2. MO disaggregation-induced emission. (a) Schematic graph of MO disaggregation while adding milk that contains triglyceride (fat). (b) 19F NMR of MO with various concentrations of extracted triglyceride. (c) Dynamic radius of MO-milk composites.

Before applying MO to real-life fat measurement, we need to confirm that it only responds to fat, not to any other substances in milk. Moreover, MO should not have any prejudice against milk with various origins. To fully explore its applicability, we tested MO in diversified milk samples, which are readily available products collected in local markets (Figure 3). They were consistently homogenized and labelled with exact amounts of all ingredients. In each brand we secured as many types of milk as possible, thus ensuring consistent milk processing procedures and largely similar ingredients. By measuring their fluorescence intensity and correlate 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). Figure 3 plots 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 the linear line, indicating that MO can be applied universally to various types of milk and their origins (Australia, New Zealand, Thailand, etc.) do not affect fat measurement.

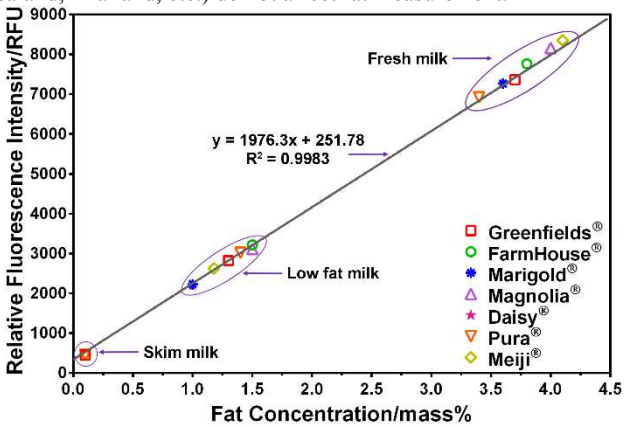



Figure 3. Selectivity test of MO. Linear line is derived from one type of milk (Greenfields®). All other data sets are plotted on the graph based on the respective fat concentration and fluorescence intensity of each type of milk.

Furthermore, Table 1 clearly exhibits that among all the 16 types of milk from 7 brands, their protein amounts range from 3% to 5%, while the carbohydrate level has an even higher range of 4.8% to 7.0%. Nevertheless, none of these two species pose any disturbance to the fluorescence measurement of fat amount. A parallel comparison among all the skim milk, low fat milk and fresh milk could clue us more information. For instance, skim milk, though all of them contain 0.1% fat, their protein concentrations vary from 3.2% to 4.0% while their carbohydrate concentrations vary from 4.2% to 6.9%. This dramatic difference within other ingredients does not affect fat measurement. Similarly, protein amount in low fat milk vary from 3.2% to 5.0% while carbohydrate amount vary from 3.9% to 7.0%; among the fresh milk, protein amount vary from 3.2% to 4% while carbohydrate vary from 3.9% to 5.0%. We can thus conclude that neither protein nor carbohydrate would affect fat measurement. This demonstrates remarkable selectivity and versatility of MO towards milk fat (Figure S5b, Table S2-S9).

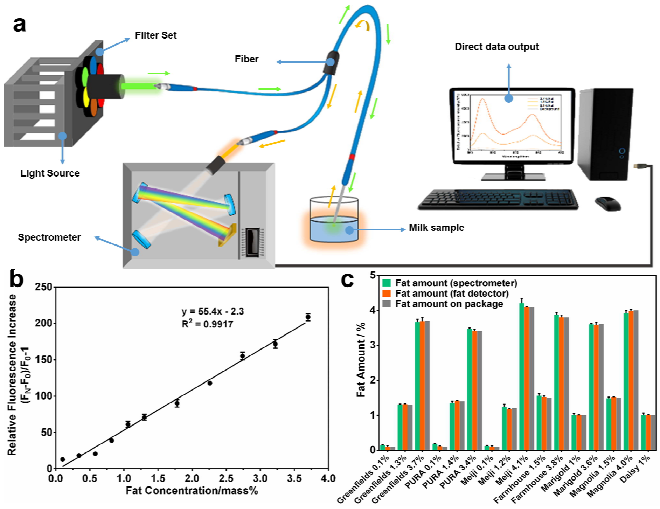



Figure 4. Construction of rapid quantitative milk fat detector. (a) Scheme of each component of milk fat detector. (b) Dose-dependent linear response graph of fluorescence intensity towards fat concentration measured by the milk fat detector. (c) Comparison chart of fat amounts acquired from spectrometer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

Prior to MO, no fluorescence milk fat sensor has been reported, not to mention any practically applicable and customer-friendly fluorescence milk fat detector. 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 (Figure 4a). For experimental purpose, 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 fluorescence signals. The optical fibre thus transfers fluorescence signals to a grating fluorescence detector that instantly separates incoming light into whole spectrum (300-1100 nm) and transmits detected photon numbers to a computer, which draws the corresponding spectra (Figure S6). Figure 4b is the linear response derived from the spectra of fat detector. Its $R^2$ value is comparable with that acquired from a complicated spectrometer, indicating that the approach to simplify the fat detection process and cost does not affect the sensitivity or accuracy. Moreover, Figure 4c shows comparison between fat amounts from all 16 types of milk measured by either spectrometer or fat detector. It is clear that both values are highly consistent with the fat amounts labelled on the package.

In summary, through the fluorescence image-based hyper-throughput screening approach, we have identified MO as a novel and unprecedented milk fat sensor, which exhibits remarkable fluorescence turn-on feature towards fat content. Its fluorescence intensity is following a perfect linear dose-dependent feature with regards to fat amount, which greatly facilitates milk fat quantitation. The sensing process is based on 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 of wide application to milk from various origins with various quality. The combination of MO with a simple fluorescence detector has opens a new window to practically apply various fluorescence sensors into 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.

## Notes and references

[a] Department of Chemistry & MedChem Program of Life Sciences Institute, National University of Singapore, 117543; Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), 138667, Singapore

[b] Singapore Peking Oxford Research Enterprise (SPORE), Environmental Research Institute (NERI), 5A Engineering Drive 1, #02-01, 117411, Singapore

[c] Department of Chemistry, Indian Institute of Technology-Kanpur

Email: chmcyt@nus.edu.sg

† We acknowledge the financial support from the Singapore-Peking-Oxford Research Enterprise (SPORE, COY-15-EWI-RCFSA/N197-1).

1. R. G. Jensen, A. M. Ferris and C. J. Lammi-Keefe, *Journal of dairy science*, 1991, **74**, 3228-3243.
2. R. G. Jensen, *Journal of dairy science*, 2002, **85**, 295-350.
3. D. L. Palmquist, A. D. Beaulieu and D. M. Barbano, *Journal of dairy science*, 1993, **76**, 1753-1771.
4. R. G. Jensen, J. G. Quinn, D. L. Carpenter and J. Sampugna, *Journal of dairy science*, 1967, **50**, 119-126.
5. C. De Jong and H. T. Badings, *Journal of High Resolution Chromatography*, 1990, **13**, 94-98.
6. T. Sato, S. Kawano and M. Iwamoto, *Journal of dairy science*, 1990, **73**, 3408-3413.
7. H. Soyeurt, P. Dardenne, F. Dehareng, G. Lognay, D. Veselko, M. Marlier, C. Bertozzi, P. Mayeres and N. Gengler, *Journal of dairy science*, 2006, **89**, 3690-3695.
8. A. P. de Silva, H. Q. Gunaratne, T. Gunnlaugsson, A. J. Huxley, C. P. McCoy, J. T. Rademacher and T. E. Rice, *Chemical reviews*, 1997, **97**, 1515-1566.
9. M. Vendrell, D. Zhai, J. C. Er and Y. T. Chang, *Chemical reviews*, 2012, **112**, 4391-4420.
10. N. Y. Kang, H. H. Ha, S. W. Yun, Y. H. Yu and Y. T. Chang, *Chemical Society reviews*, 2011, **40**, 3613-3626.
11. S. W. Yun, N. Y. Kang, S. J. Park, H. H. Ha, Y. K. Kim, J. S. Lee and Y. T. Chang, *Accounts of chemical research*, 2014, **47**, 1277-1286.
12. A. Villar, E. Gorritxategi, E. Aranzabe, S. Fernandez, D. Otaduy and L. A. Fernandez, *Food chemistry*, 2012, **135**, 2756-2760.
13. D. Zhai, B. K. Agrawalla, P. S. Eng, S. C. Lee, W. Xu and Y. T. Chang, *Chemical communications*, 2013, **49**, 6170-6172.
14. Y. Takaoka, T. Sakamoto, S. Tsukiji, M. Narazaki, T. Matsuda, H. Tochio, M. Shirakawa and I. Hamachi, *Nature chemistry*, 2009, **1**, 557-561.
15. W. Xu, T. H. Kim, D. Zhai, J. C. Er, L. Zhang, A. A. Kale, B. K. Agrawalla, Y. K. Cho and Y. T. Chang, *Scientific reports*, 2013, **3**, 2255.
16. D. Zhai, W. Xu, L. Zhang and Y. T. Chang, *Chemical Society reviews*, 2014, **43**, 2402-2411.