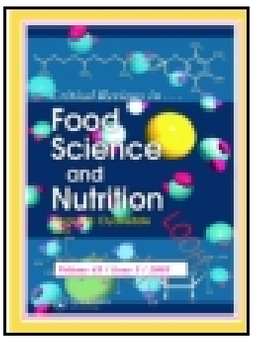

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Potential applications of luminescent molecular rotors in food science and engineering

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ABSTRACT

Fluorescent molecular rotors (MRs) are compounds whose emission is modulated by segmental mobility; photoexcitation generates a locally excited (LE), planar state that can relax either by radiative decay (emission of a photon) or by formation of a twisted intramolecular charge transfer (TICT) state that can relax nonradiatively due to internal rotation. If the local environment around the probe allows for rapid internal rotation in the excited state, fast non-radiative decay can either effectively quench the fluorescence or generate a second, red-shifted emission band. Conversely, any environmental restriction to twisting in the excited state due to free volume, crowding or viscosity, slows rotational relaxation and promotes fluorescence emission from the LE state. The environmental sensitivity of MRs has been exploited extensively in biological applications to sense microviscosity in biofluids, the stability and physical state of biomembranes, and conformational changes in macromolecules. The application of MRs in food research, however, has been only marginally explored. In this review, we summarize the main characteristics of fluorescent MRs, their current applications in biological research and their current and potential applications as sensors of physical properties in food science and engineering.

Abbreviations: BODIPY: boron-dipyrromethene; CCJV: 9-(2-cyano-2-hydroxy carbonyl)-vinyl julolidine; CPVDA: p-[(2-cyano-2-propanedio ester) vinyl] dimethylaniline; DMABN: 4,4-dimethylaminobenzonitrile; DCVJ: 9-(dicyanovinyl)-julolidine; DASPI: (dimethylamino)-styryl-1-methylpyridinium iodide; GRAS: generally recognized as safe; MR: molecular rotor; SY: sunset yellow; TEG: triethyleneglycol ester

KEYWORDS

Luminescence spectroscopy; molecular rotors; free volume; molecular crowding; micro-viscosity; TICT state

Introduction

Luminescence spectroscopy due to its convenience and versatility is commonly used to study the molecular properties and functionality of proteins, lipid membranes, and nucleic acids in the biological sciences (Christensen et al., 2006; Corradini and Ludescher, 2015; Corradini et al., 2016; Karoui and Blecker, 2011; Strasburg and Ludescher, 1995). The environmental sensitivity of organic chromophores to the physical and chemical properties of their local surroundings, such as pH, ionic strength, polarity, hydrogen bonding, or matrix mobility (Jameson, 2014; Turro et al., 2010), make these fluorophores excellent *in situ* or even *in vivo* molecular sensors causing minimal or no perturbation to the system. In recent years, the use of spectroscopic techniques in food applications has significantly increased, due, in part, to several factors: (a) the identification of suitable Generally Recognized as Safe (GRAS) fluorophores (Corradini and Ludescher, 2015; Corradini et al., 2016), (b) instrumental improvements, and (c) advances in chemometric methods that facilitate data acquisition, interpretation and implementation of spectrophotometric techniques in food matrices (Garcia-Canas et al., 2012; Skov et al., 2014). However, in our opinion, luminescence spectroscopy as a tool to study food structure and food properties is still significantly underutilized. This work reviews current and potential applications of a specific class of optical probes, known as luminescent molecular rotors (MRs), in the food sciences. MRs, i.e., compounds with molecular structures that display internal motion, are numerous and encompass molecules that exhibit internal rotation to various degrees, e.g., partial rotation or 360° (Kottas et al., 2005). The vast diversity of MRs that have been currently identified or synthesized stem from differences in their structure, driving mechanism (e.g., thermal energy vs. external forces), function, and state (e.g., free in solution, included within solids, or surface mounted) (Bracco et al., 2015; Comotti et al., 2010, 2014a, 2014b; Vogelsberg and Garcia-Garibay, 2012; Kottas et al., 2005). During the last two decades important advances in the study of MRs permitted to understand and harness their driving forces, increase their functionality and assemble them into sensors and molecular machines. Additional information on the characteristics, operation and

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applicability of different kinds of MRs, both in solution and solid state, can be found in comprehensive reviews by Kottas et al. (2005), Abendroth et al. (2015), Comotti et al. (2016), Erbas-Cakmak et al. (2015), and Lensen and Elemans (2012). In this review, we particularly focus on a narrow subset of molecules endowed with fast dynamics and viscosity-sensitive photophysical properties. These luminescent MRs exhibit a remarkable sensitivity to molecular crowding, free volume, and viscosity and consequently have been proposed as effective intrinsic sensors of quality attributes, particularly of physical properties, in foods (Corradini and Ludescher, 2015; Corradini et al., 2016; Haidekker and Theodorakis, 2010).

Manufactured foods are complex and often multi-phased systems (Mezzenga et al., 2005) whilst others such as fruits and vegetables are consumed raw or minimally processed. Regardless, many foods are presented to the consumer after extensive modification, resulting in the development of unique microstructural characteristics. Accordingly, a thorough understanding of the influence of microstructure and composition on organoleptic and physicochemical properties of the foods is crucial to sustain product quality and shelf life (McClements, 2007). Luminescence techniques based on the use of environmentally sensitive fluorescent probes that function as MRs can serve as versatile tools for such a purpose due to their ability to report on various physical properties of their surrounding environment in a noninvasive and non-disruptive way. Luminescent MRs exhibit internal segmental mobility in the excited state that can quench fluorescence. The basis for their environmental sensitivity is hindrance of intramolecular rotation (Haidekker et al., 2010); therefore, interactions of a MR with an interface, a membrane, or a protein, or exposure to variable degrees of molecular crowding will increase the fluorescence emission intensity, quantum yield and lifetime. A MR may be embedded into a static system, i.e., a food or a cell, and report on properties such as micro (and bulk) viscosity. Alternatively, MRs can be operationalized into a dynamic industrial setting to monitor changes throughout the processing line and/or to obtain measurements of quality attributes over time at a specific point. Fluorescence lifetime imaging microscopy (FLIM) of MRs can also be used to map the spatial distribution of viscosity in cellular environments (Loison et al., 2013; Suhling et al., 2012) and model systems (Dent et al., 2015; Wu et al., 2013). An overview of the basic operational principles of fluorescent MRs and their current applications in the biological sciences will be presented first, followed by a discussion of the current and potential uses of this class of luminescent probes in food science and engineering.

Fluorescent molecular rotors

An ideal viscosity-sensitive MR has the following attributes: (a) a large Stokes shift (the difference in wavelength between the excitation and emission spectra) that results in good differentiation of excitation and emission spectra, (b) high sensitivity to structural rigidity of the surrounding media, (c) high brightness (Sutharsan et al., 2010a), and (d) no coupling of the response to structural rigidity with sensitivity to other physical and chemical properties such as polarity. Table 1 illustrates some of the major groups of MRs that have been identified and studied.

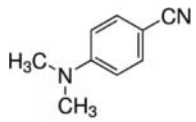
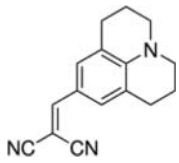
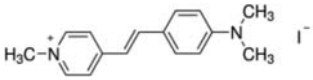
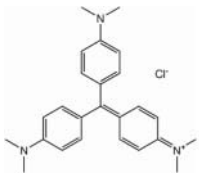
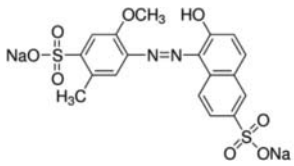
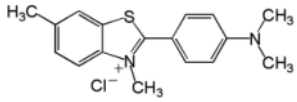
Mode of action

MRs, molecules that can undergo an intramolecular segmental rotational (twisting) motion following photoexcitation (Amdursky et al., 2012), typically consist of an electron donor and an electron acceptor group connected by a conjugated network of alternating pi (π) and sigma (σ) bonds (Fig. 1). Photoexcitation forms a locally excited (LE) intramolecular charge transfer state (ICT) in which an electron is internally transferred from the electron donor to the acceptor (Grabowski et al., 2003). Because of their conjugated system of π orbitals, MRs have a planar ground state configuration. While the initial ICT state formed on photoexcitation is also planar, electrostatic forces resulting from the charge separation induce twisting of the sub-groups on the molecule relative to one another; the resulting non-planar configuration, the twisted intramolecular charge transfer (TICT) state, has a lower excited state energy. Relaxation from the TICT state involves either radiative decay of a photon, giving rise to a bathochromic shift in the emission band, or non-radiative decay via energy dissipating vibrational motions (Rettig, 1982; Allen et al., 2005) (Fig. 2). The rate of formation of the TICT state from the LE charge transfer state is dependent on the microenvironment of the matrix, that is, on the free volume around the MR. Molecular crowding, or high medium microviscosity, sterically restricts the formation of the TICT state, thus favoring persistence of the locally excited ICT state; relaxation from the LE state involves direct photon emission, known as fluorescence. The energy gap between the newly formed TICT state and the ground state plays an important role in the subsequent relaxation behavior of the TICT state (Haidekker and Theodorakis, 2010). If the TICT-ground state energy gap is small, the TICT state relaxes directly to the ground state by fast non-radiative decay; in this case, there is only a single emission band reflecting radiative decay from the LE state (Haidekker and Theodorakis, 2010) (Fig. 2A). If the energy gap is sufficiently large, the TICT state can relax by radiative emission, resulting in a second emission band that is red-shifted from the LE fluorescence band (Haidekker and Theodorakis, 2010) (Fig. 2B). Such dual band emission (Haidekker et al., 2010) is exemplified by 4,4-dimethylaminobenzonitrile (DMABN), which belongs to the class of benzonitrile MRs (Haidekker et al., 2005). In either class of MRs, emission from the locally excited ICT state is sensitive to the physical state of the local environment: any increase in molecular crowding/microviscosity increases the probability of emission from the LE state and thus increases the intensity of the LE emission band.

Sensitivity to free volume (and molecular crowding)

The free volume around the MR thus modulates the rate of formation of the TICT state and thus the fluorescence intensity of the LE state (Paul and Samanta, 2008): LE emission intensity is low in fluid and high in rigid conditions. Since solution viscosity, which reflects molecular crowding and free volume (Doolittle, 1952), is easy to modify and measure, the environmental sensitivity of MRs has been mainly evaluated and expressed as sensitivity to solution viscosity.

Table 1. Classification, examples, structure and photophysical properties of the major groups of MRs identified to this date.

Type	Examples	Typical Structure	Excitation Wavelength (nm)	Emission Wavelength (nm)	Ref.
Benzonitrile-based fluorophores	DMABN		290	342 / 460 *	(Haidekker et al., 2005)
Benzylidene malononitriles	DCVJ		489	505	(Haidekker et al., 2005; Jee et al., 2009)
Stilbenes	p-DASPI		430	625	(Jee et al., 2010)
Triarylmethane dyes	Crystal Violet,		590	630	(Haidekker et al., 2005)
Azo dyes	Fast Green Allura Red,		470 /600 520	515 /660 590	(Kashi et al., 2015; Du et al., 2014)
Benzothiazole	Citrus Red Thioflavin T		520 420	610 485	(Vus et al., 2015)

*Dual fluorescence bands

Loutfy and Arnold's (1982) earliest work, which studied the relationship between viscosity and fluorescence quantum yield in a wide range of solvents, suggests that, in low viscosity solvents, hydrodynamics can clearly drive the free rotor effect of MRs. However, in higher viscosity solvents, hydrodynamic predictions are insufficient; instead, free-volume is the primary influence on the segmental relaxation of the probe. They also observed an increase in MR lifetime with solvent viscosity, from liquid ethyl-acetate, a relatively lower viscosity solvent (11 picoseconds), to viscous glycerol, a relatively medium viscosity solvent (500 ps), to rigid glasses, a relatively higher viscosity matrix (3600 ps). Accordingly, they proposed that the relaxation from the excited state is dependent on the solvent viscosity. A proportional relationship has been established between solvent viscosity and fluorescent quantum yield. The Förster-Hoffmann equation (Loutfy and Arnold, 1982) describes the following power law relationship between the fluorescent quantum yield, Φ_F, and the viscosity (η) of the solution:

$$\log\Phi_F = C + x\log\eta \quad (1)$$

where C is a dye-dependent constant and x is a constant related to dye-solvent interactions. Since fluorescence emission

Figure 1. Schematic diagram of molecular rotor structure that adopts an "electron donor-π-electron acceptor" (A); 'D' represents electron donor segment, 'A' represents electron acceptor segment; the two segments are connected via a π-conjugation unit. Adapted from Haidekker and Theodorakis (2007).

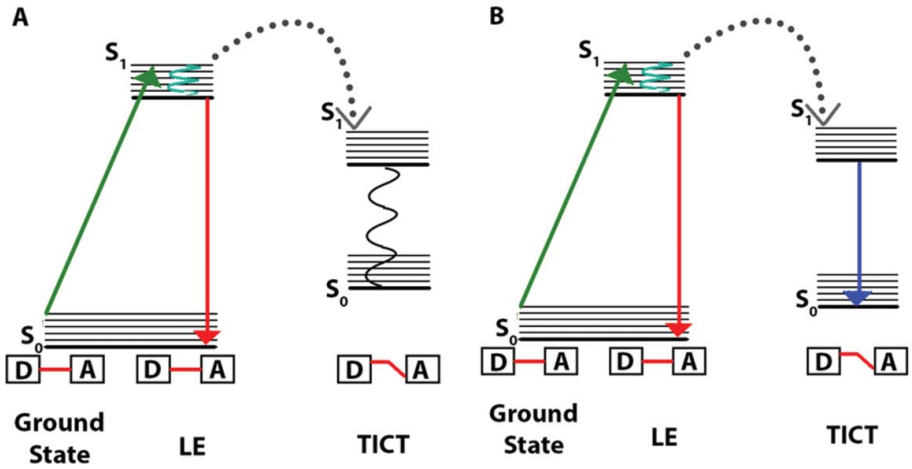


Figure 2. Jablonski diagram for single (A), and dual band (B) MRs. Photoexcitation promotes a MR from ground state (S0) to the excited state (S1). The series of parallel lines represent vibrational states. (A) In a single band MR, S1 may relax by photon emission from the LE state (red arrow) or by non-radiative decay from the TICT state (curved line). (B) In a dual band MR, S1 may relax by photon emission from either the LE or the TICT states (red and blue arrows, respectively). Adapted from Haidekker and Theodorakis (2010).

intensity and quantum yield are proportional, the relationship between the measured fluorescence intensity and viscosity can be reworked from Eq. (1) and is often expressed by the following power law model (Haidekker et al., 2010):

$$I_F = \alpha \eta^x \tag{2}$$

where α is usually considered a measure of the probe's brightness and x a measure of its sensitivity to local viscosity. The sensitivity of novel MRs to viscosity is quantified by the parameter x and is compared to reported values of commonly used MRs. It should be noted that the highest theoretical value for x is considered to be 0.66 (Sutharsan et al., 2010a). Sensitivity values of novel and commonly used MRs in glycerol-based solutions have been reported in the range of 0.25 to 0.6. Table 2 provides selected examples of the values of parameters x and α for several known and recently reported MRs. (See supplementary materials for a complete list of sensitivity parameters for MRs.)

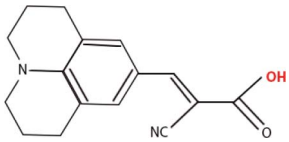
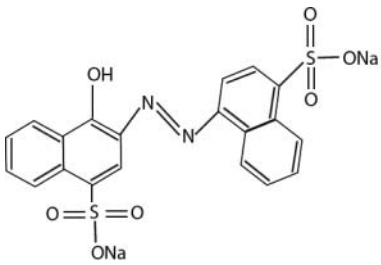
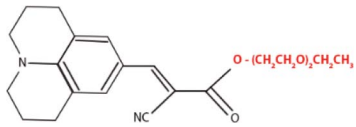
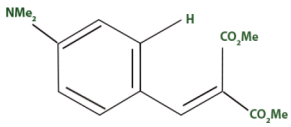
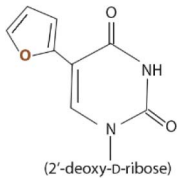
Sensitivity to polarity

Solvent polarity generally does not influence the quantum yield of MRs; instead, polarity shifts the emission peak wavelength (Loutfy and Law, 1980) or broadens the spectrum (Allen et al., 2005). Haidekker et al. (2005) investigated the effect of polarity and microviscosity on the fluorescence emission of 9-(Dicyanovinyl)-julolidine (DCVJ), 9-(2-Cyano-2-hydroxy carbonyl)-vinyl julolidine-triethyleneglycol ester (CCVJ-TEG), and DMABN. The photophysical properties DCVJ and CCVJ-TEG are independent of polarity but are highly sensitive to changes in viscosity (Haidekker et al., 2005) while DMABN is an exception as polarity modulates both the Stokes shift and emission intensity. Since the MR is composed of an electron donor (often containing oxygen or nitrogen) conjugated to an electron acceptor (for example, nitrile) (Sutharsan et al., 2010b), the presence of a weaker electron donor (e.g., methoxy, phenyl, or naphthyl group) causes a hypsochromic shift in both excitation and emission peak locations. Additionally, replacing the electron acceptor group with a methyl ester or phenyl sulfonyl group increases the quantum yield, which has been attributed to the dipole moment of the molecule (Sutharsan et al., 2010b). A recent study on DCVJ and CCVJ revealed a significant difference between viscosity sensitivity of these fluorophores in polar protic and polar aprotic solvents (Howell et al., 2012); the differences were attributed to the ability of the solvent to form hydrogen bonds. It is thus evident that the nature of the medium under study must be taken into consideration when selecting a fluorophore as a viscosity sensor.

Structure-property relationships

The seminal work by Loutfy and Law (1980) on MRs investigated the photophysical properties and conformational changes of intramolecular charge transfer in p-N,N-dialkylaminobenzylidenemalononitriles. They concluded that as the conformational rigidity of the electron donor, i.e., the NR_2, group increases, the quantum yield also increases due to a reduction in the free rotor effect. These authors also suggested that a MR with a smaller NR_2 group exhibits a lower quantum yield since this group will require less free volume to move than bulkier moieties. The influence of the molecular footprint of the rotor on the quantum yield (fluorescence intensity) and viscosity sensitivity was also studied by incorporating substituents on naphthalene-based (Sutharsan et al., 2010b; Brummond and Kocsis, 2015), phenyl-based (Sutharsan et al., 2010b), and boron -dipyrromethene (BODIPY) MR probes (Bahaidarah et al., 2014). The addition of substituents usually resulted in increases in quantum yield or fluorescence intensity and reduced viscosity sensitivity. This phenomenon is explained by the location of the donor and acceptor groups; when the groups are placed in close proximity, their rotational ability decreases and the energy level of the TICT state increases, resulting in fluorescence without significant passage through the TICT state; consequently, these substituted MRs exhibit higher fluorescence intensity and less viscosity sensitivity (Sutharsan et al., 2010b; Brummond and Kocsis, 2015; Kocsis et al., 2015). Increased viscosity sensitivity was only reported when the substituents were attached at remote sites on the BODIPY fluorophore, which

Table 2. Reported values for parameters 'x' and 'α' (Eq. 2) for selected MRs.

Molecular Rotor	Structure	x: Viscosity Sensitivity	α: Brightness	Reference
CCVJ		0.54	147	(Sutharsan et al., 2010b)
Azo Dye (Azorubine)		0.38	–	(Kashi et al., 2015)
CCVJ derivative (addition of ethylene glycol)		0.61	–	(Haidekker et al., 2004)
Dimethyl 2-(4-(dimethylamino)benzylidene) malonate		0.52	17	(Sutharsan et al., 2010b)
Nucleoside (pyrimidine – furan)		0.40	–	(Sinkeldam et al., 2011)

did not modify the space available for rotation (Bahaidarah et al., 2014). Sutharsan et al. (2010b) also reported that a modification of the π-conjugation system can increase the electron density between the electron donor and acceptor groups, which increases the quantum yield; however, steric hindrances could limit this increase.

Sinkeldam et al. (2011) elucidated the role of aryl-aryl bonds in the fluorescence of MRs based on the response of structurally modified nucleosides to changes in viscosity. In this study, pyrimidine was attached in different ways to aromatic moieties of different sizes. Attachment of the pyrimidine to furan or thiophene rings by a single rotatable bond resulted in similar viscosity sensitivity. By replacing the single bond between the two aromatic ring structures with a conjugating ethynyl bond, the emission intensity was reduced. Fusing the pyrimidine to a thiophene heterocycle completely inhibited emission in response to viscosity changes (Sinkeldam et al., 2011) due to elimination of the rotatable linkage.

Further changes in the structure of the MR have been introduced to increase functionality and performance. Traditional rotors, e.g., DCVJ, normally exhibit short excitation and emission wavelengths and small Stokes shifts. Typically, a larger Stokes shift is desirable in fluorescence measurements, to allow greater differentiation between the excitation and emission peaks; a significant overlap of the two spectra hinders detection of maximum fluorescence. To overcome this drawback, a thiophene unit was added to aryl–dicyanovinyl fluorescent MRs, which resulted in larger Stokes shifts and red-shifted emission, facilitating differentiation of the MR emission from that of the background (Shao et al., 2011). Self-calibrating MRs composed of an internal reference, a viscosity-independent dye, attached to a viscosity sensitive unit have been synthesized (Fig. 3A) (Dakanali et al., 2012; Nipper et al., 2011; Haidekker et al., 2006). These ratiometric probes exhibit two emission bands whose intensity ratio can provide a concentration-independent self-calibrating measurement of the viscosity. In these ratiometric rotors, the emission spectrum of the reference dye (viscosity-independent moiety) overlaps the excitation spectrum of the MR probe (viscosity dependent moiety) so that resonance energy transfer (RET) can occur from reference to MR (Fig. 3b); a

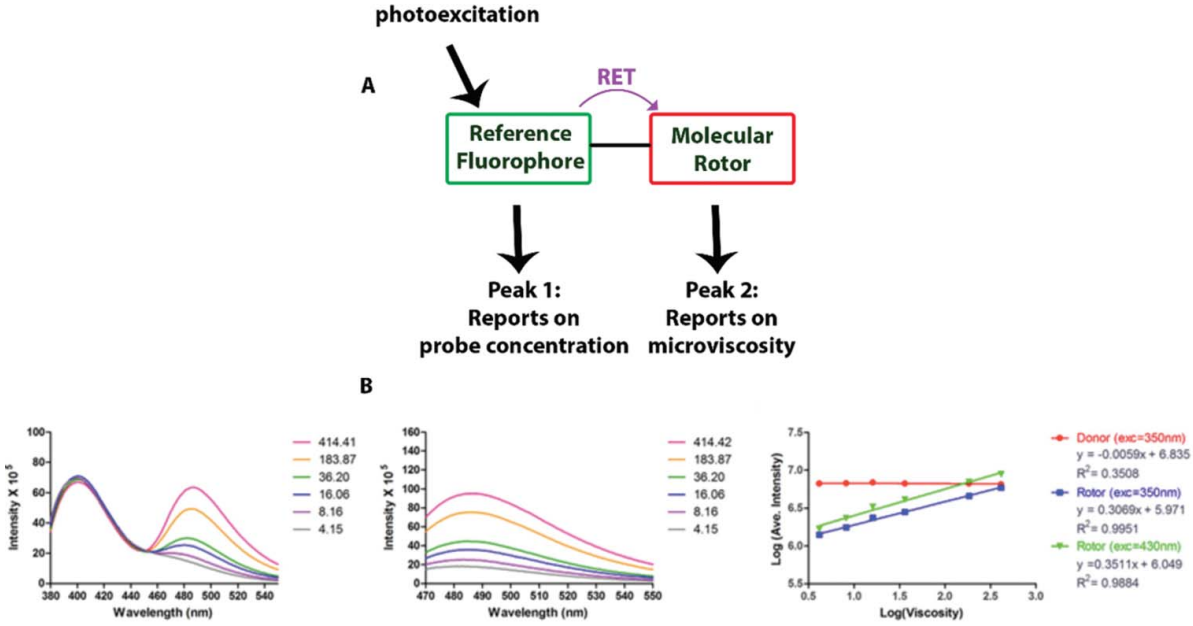


Figure 3. (A) Schematic diagram of a ratiometric probe with viscosity sensitivity. (B) Examples of fluorescence emission data (from left): ratiometric MR emission spectra, MR emission spectra alone, and viscosity sensitivity of ratiometric dyes; reproduced with permission from Dakanali et al. (2012).

single excitation wavelength thus excites both reference fluorescence directly and MR fluorescence by RET. Haidekker et al. (Dakanali et al., 2012; Nipper et al., 2011; Haidekker et al., 2006) developed a ratiometric viscosity sensor by covalently attaching coumarin-based reference dyes to thiophene or aniline based MRs. Conjugated porphyrin dimers (Kuimova, 2012a) and a pentamethine cyanine dye substituted with an aldehyde group at its meso position (Peng et al., 2011) have also been used to obtain fluorescent ratiometric viscosity measurements.

Readers are referred to the review of Uzhinov et al. (2011) for further details on the influence of chemical structure on the photophysical properties of various MRs and to Haidekker and Theodorakis (2010) for an in-depth review of these photophysical properties.

Applications of fluorescent molecular rotors in biological sciences

MRs have found numerous applications in the biological sciences (Suhling et al., 2012; Kuimova, 2012b; Peng et al., 2011; Wang and Ma, 2010; Kuimova et al., 2009; Battisti et al., 2013; Levitt et al., 2011; Lopez-Duarte et al., 2014) and specifically in molecular biology due to the need of sensors with the ability to report on physical properties such as cytoplasm viscosity or membrane fluidity without perturbing normal system functions. Since spectroscopy of MRs is minimally invasive, it allows for the direct investigation of dynamic properties of proteins, cellular organelles, membranes, and the cytoplasm within living cells and tissues. This section summarizes some of the most salient reported applications of MRs in biological systems.

Biofluids and intracellular viscosity

Blood plasma viscosity can be used for early diagnosis of disease (Baskurt and Meiselman, 2003). Haidekker et al. (2002) and Akers et al. (2005) correlated plasma viscosity and fluorescence emission intensity of julolidine-based MRs. The latter work compared fluorescence-based viscosity assessment to a conventional mechanical method (cone-plate rheometry) in terms of methodology and precision. The results show slightly greater scatter when blood viscosity was determined by cone-and-plate rheometry (<7.6%) compared to fluorescence spectroscopy using MRs (<6%), suggesting that non-mechanical sensing can be, at least, equally effective and reliable as mechanical-based methods.

The microviscosity in the cellular cytoplasm is of critical importance to optimal cell functioning due to its effect on processes such as protein folding and other biochemical reactions (Hall and Minton, 2003; Ellis, 2001). The *meso*-substituted BODIPY (Levitt et al., 2011) and *meso*-substituted 4,4′-difluoro-4-bora-3a,4a-diaza-s-indacene (Kuimova, 2012b) MRs have been used to report on microviscosity in live SK-OV-3 human carcinoma cells. A ratiometric viscosity sensitive probe synthesized by Dakanali et al. (2012) selectively localizes in the cytoplasm and remains in the aqueous phase. Fluorescence lifetime imaging microscopy (FLIM) is used to map the spatial distribution of MR lifetimes (which are independent of dye concentration) that can be correlated with medium viscosity; it is thus possible to map viscosity distribution across heterogeneous cellular environments. A scheme of a FLIM set up using MRs is presented in Fig. 4. The use of fluorescence microscopy enables spatial and time-based tracking of properties of interest (Suhling et al., 2012; Festy et al., 2007). Intracellular viscosity in living HeLa cells was estimated with FLIM using a *meso*-substituted BODIPY-C_{12} MR as a probe (Thompson et al., 2015); Fig. 5 illustrates the correlation between longer fluorescence lifetime and higher medium viscosity for this probe.

The same technique was used to measure viscosities within the compartments of a primitive cell model consisting of a coacervate core (mimicking a reaction center) coated by oleic acid (mimicking a cell membrane) using Kiton Red and BODIPY-

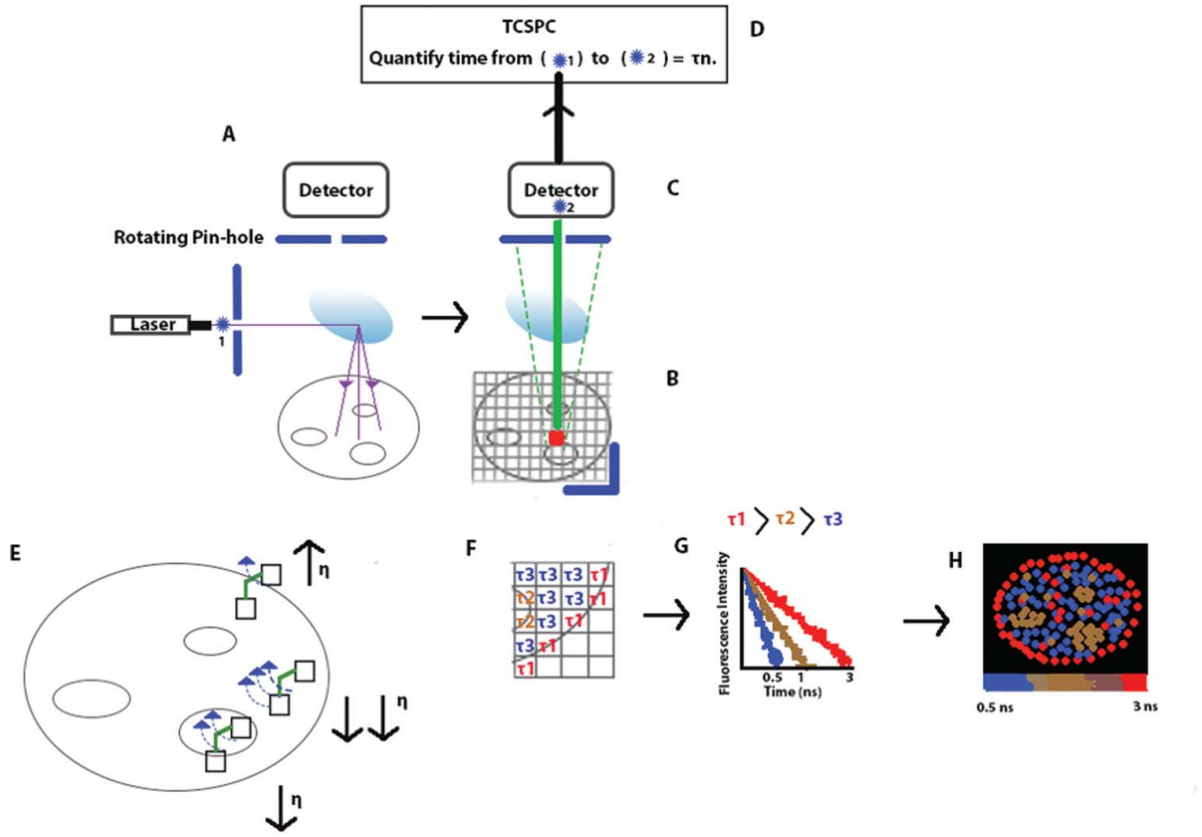


Figure 4. Schematic diagram of FLIM to obtain a high-resolution viscosity map of a biological sample. (A) High-resolution fluorescence images are obtained using a confocal scanning microscope. A pulsed laser beam (purple line) is directed towards a dichromic mirror that reflects the light onto a biological sample that has been stained with MR. (B) Sample perceived as multiple pixelated regions; (C) Emission from a single pixel at a time is selectively allowed to pass to the detector through a rotating pin-hole structure while light emitted from other pixels is blocked. (D) A time correlated single photon counting (TCSPC) unit quantifies the duration of the emission; (F) Specific lifetimes (τ) are allocated to each individual pixel. (E) MR decay pathways are microviscosity dependent; increased viscosity (η) hinders rotation (single rotation arrow); longer fluorescence lifetime (τ1); lower viscosity facilitates rotation; shorter fluorescence lifetime (τ3). (F-H) Software is used to compile all τ values into a viscosity map using a false color scale.

C_{10}, respectively (Thompson et al., 2014; Ferri et al., 2016). Fluorescence lifetimes were also found to be organelle-specific within cells and have been used to estimate viscosity values (Levitt et al., 2009). Another MR, a conjugated porphyrin dimer, was exploited for its unique ability to function as both a photosensitizer that induces cell death upon its irradiation, and a reporter of the resultant dynamic changes in intracellular viscosity (Kuimova et al., 2009). Photoinduced cell death resulted in an increase in fluorescence intensity, illustrating the expected intracellular viscosity increase (~300 cP). This rotor is thus able to report on real-time changes in membrane rigidity as a function of irradiation as well as the longevity of the treated cell.

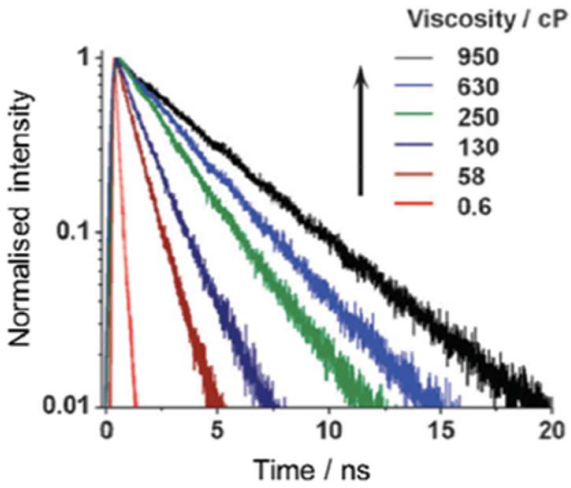


Figure 5. Time resolved fluorescence of BODIPY-C10 in methanol-glycerol mixtures of varying viscosity. Reprinted with permission from Thompson et al. (2015).

Macroscopic membrane properties

Diffusion, packing and permeability are dependent on cell membrane properties such as viscosity and fluidity (van der Meer, 1984). The functionality of membrane-bound receptors and enzymes, for example, depends on the membrane viscosity (Dunham et al., 1996; Kung and Reed, 1986) and alterations in membrane properties were found to adversely affect enzyme functionality, active-transport and facilitated diffusion across the membrane, as well as binding of receptors (Rohrbach and Timpl, 1993). Numerous health disorders have also been associated with cell membrane viscosity changes, including cardiovascular diseases (Luneva et al., 2007), cell malignancy (Shinitzky, 1984), hypertension (Kearney-Schwartz et al., 2007), hypercholesterolemia (Deliconstantinos et al., 1995), diabetes (Salazar Vázquez et al., 2008), and Alzheimer's disease (Hou et al., 2005). Nipper et al. (2008) investigated changes in the viscosity of a liposome model membrane using a membrane-entrapped

farnesol ester of MR (2-carboxy-2-cyanovinyl)-julolidine, FCVJ. This rotor was found to be sensitive to changes in the membrane viscosity upon addition of viscosity-increasing (e.g., cholesterol) and viscosity–decreasing (e.g., longer chain alcohols) agents.

Lipid membranes and liposomes undergo a temperature-dependent phase change, assuming a gel state below the phase transition temperature and a liquid crystalline state above that temperature (Lentz et al., 1976). The use of MRs has been explored to distinguish between liquid-ordered (L_o) and liquid disordered (L_d) phase domains in cell membranes; L_o domains have a relatively higher viscosity than L_d domains and play a role in regulating membrane trafficking and signal transduction (Anderson and Jacobson, 2002; Simons and Ehehalt, 2002). An auramine-based MR, conjugated to a cholesteryl group that acts as an anchor into a bilayer lipid membrane was used with epi-fluorescence microscopy to report on the spatial distribution of lipid membrane viscosity. The images of individual gel-state and liquid-crystalline state liposomes showed regions of bright and weaker green emission, respectively. Similarly, a synthesized membrane with phase-separated L_o and L_d domains showed both high and low-emitting regions, which have been allocated to L_o and L_d domains based on the viscosity-dependent fluorescence intensity of the auramine MR probe (Fig. 6) (Yasuhara et al., 2008). L_o and L_d membrane domains were also investigated using *meso*-substituted BODIPY–C_{10} and –C_{12} aliphatic chains (Wu et al., 2013) by correlating time-resolved fluorescence decays with temperature-dependent phase state changes in the lipid bilayers. The rotor proved an effective identifier of the two different order regions based on fluorescence lifetimes.

Protein aggregation, degradation and conformational changes

The abnormal aggregation of proteins or polypeptides into amyloid fibrils (Ramírez-Alvarado et al., 2000) has been associated with numerous diseases such as neurological disorders, type II diabetes and systemic amyloidosis (Pulawski et al., 2012). MRs have been used to identify the presence and formation of amyloids (Thompson et al., 2015; Vus et al., 2015) and the effect that therapeutic strategies might have on their stability (Amdursky et al., 2012). For example, Cy3 MR was used to quantify the steric hindrance to the MR at the various stages of amyloid fibril formation, which allowed for comparison of the different amyloid fibrils that resulted from different protein aggregates (Thompson et al., 2014). A variety of amyloid binding molecules with inherent fluorescent properties have been developed. Among them, thioflavin T derived MRs (Vus et al., 2015) and novel probes produced by π-conjugation of a diakyl amino group with a 2-cyanoacrylate unit (Sutharsan et al., 2010a) have potential as new diagnostic tools for investigation of amyloid-based diseases. Additionally, conjugates of MRs and protein ligands have been proposed to track in real-time the degradation of proteins in living cells; upon ligand binding to the target protein, molecular crowding limits the segmental motion of the MR increasing emission intensity; this intensity decreases upon protein degradation (Yu et al., 2016).

Other applications

Small fluorescent MRs have also been used to report on the viscoelastic properties of microbubbles. The fluorophores bind to the surface of the microbubbles and their fluorescence emission and lifetime are modulated by the local free volume (Hosny et al., 2013a); spatial variations of the fluorescent signal can be correlated to stability and functionality. Expanding the applications of microbubbles into ultrasound imaging and drug delivery (Hosny et al., 2013b) requires understanding how composition and fabrication methods affect their surface properties.

These extensive applications of MRs in biological systems provide a roadmap to extend their use to food systems. In the

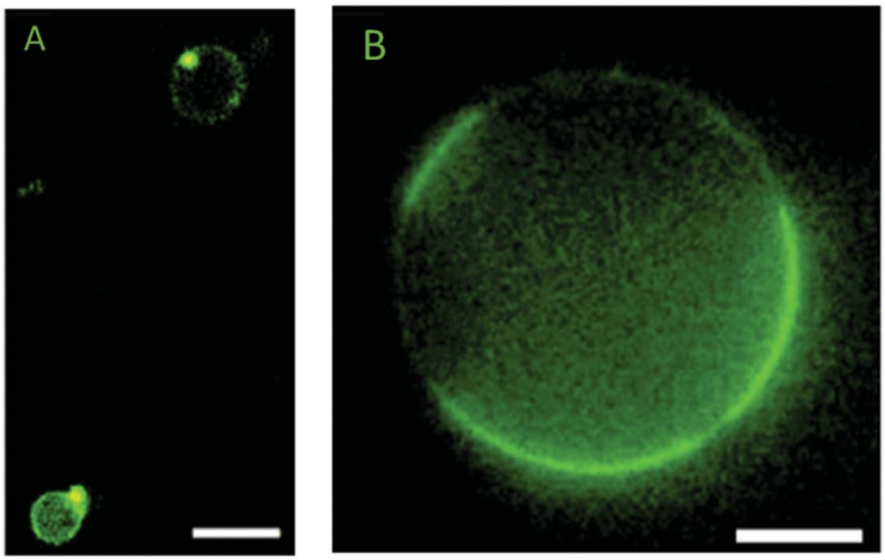


Figure 6. FLIM image of an auramine-based MR conjugated to a cholesteryl group embedded into a bilayer lipid membrane. (A) The MR probe is able to distinguish between the gel (bright emission = higher viscosity) and liquid-crystalline (weak emission = lower viscosity) phases of the liposome bilayer membrane. (B) The MR probe can also distinguish between domains in a synthesized giant liposome containing both Lo (more viscous and brighter) and Ld (less viscous and darker region). Reproduced with permission from Yasuhara et al. (2008).

following section, we discuss current research using MRs to investigate food properties as well as potential novel applications of these versatile probes.

Potential applications of molecular rotors in food science and engineering

The versatility of MRs offers the potential of direct optical measurement of physical properties related to food quality and stability. Based on the applications discussed above, MR probes can specifically contribute to understanding interactions between food components and the effect of microstructure on food properties, reporting on, for example, polymerization processes, protein degradation, colloidal stability, and phase states and transitions (Haidekker and Theodorakis, 2010; Uzhinov et al., 2011; Kashi et al., 2015). It should be noted, however, that the limited solubility, price, and especially toxicity of many conventional MRs limit their application in foods. To overcome this drawback, Corradini and Ludescher (2015) have proposed searching for MRs among the many fluorescent molecules found in foods, in order to identify and characterize a library of GRAS probes as intrinsic luminescent sensors of food quality, safety and stability.

Micro and bulk viscosity of liquid and semi-solid foods and food models

Edible azo and aryl methane dyes, including the synthetic food colors Allura Red, Azorubine, Sunset Yellow, Tartrazine, Citrus Red, Fast Green, and Brilliant Blue, are essentially non-fluorescent in fluid solution but become fluorescent in viscous solutions (Kashi et al., 2015; Corradini et al., 2016; Corradini and Ludescher, 2015). Studies of mono-azo dyes in glycerol/water solutions at constant temperature and in glycerol as a function of temperature indicate that its fluorescence intensity follows a power law behavior (Eq. 2) over variations in viscosity ranging from 1–10^4 mPa s. Values of 0.35–0.45 for the viscosity sensitivity parameter (x in Eq. 2) in these systems provides strong evidence that mono azo dyes and by implication other similar food dyes, behave as a MR (Table 2).

Kashi et al. (2015) measured the effect of increasing viscosity on the fluorescence intensity of several food dyes in aqueous model systems of identical viscosity (90 mPa s) containing either glycerol, glucose, sucrose or hydrocolloids (Fig. 7, left). They observed that the sensitivity to bulk viscosity was somewhat lower in the methyl cellulose (MC) and much lower in the carboxymethylcellulose (CMC) solutions. The authors speculated that the lower sensitivity to viscosity was due to extensive hydration and less molecular crowding around the MR in the polymeric hydrocolloid solutions. They used pyranine as a fluorescent sensor of molecular crowding based on its ability to report on the amount of water in its surroundings (Fig. 7, right) (Roche et al., 2006); this probe exhibits dual emission bands that reflect either protonated (peak ~435 nm) or unprotonated (peak ~510 nm) pyranine; the extent of deprotonation is increased by available water (by available sites for the proton). The intensity of the unprotonated pyranine band varied in a complementary manner to the effect of viscosity on the azo dye emission intensity: those solutions in which the azo dye exhibited high intensity (those containing high concentrations of glycerol, glucose or sucrose) exhibited low intensity of the unprotonated pyranine band, and thus limited hydration, while those hydrocolloid solutions with lower mono azo dye intensity exhibited high band intensity, and thus extensive hydration of the probe. Although these results explain the lack of sensitivity of azo dyes to viscosity modulate by CMC, they do not fully account for the greater effect of methyl cellulose on azo dye intensity. The authors hypothesized that differences in the sensitivity of the dye to bulk viscosity in hydrocolloid solutions may reflect the hydrodynamic size, the structure, or the intrinsic rigidity of the hydrocolloid polymer or with interactions between the probe and the polymer. The use of MRs as sensors of bulk viscosity was also reported by Akers and Haidekker (2004) who make use of CCVJ in aqueous colloidal solutions of dextran and hydroxyethyl starch and by Dragan et al. (2014) who designed two novel MRs, PicoGreen and SYBR Green, and tested them in collagen solutions. These MRs effectively responded to the bulk viscosity of these solutions of macromolecules. It is important to note that MRs report on the properties of solutions at the molecular level and their potential use as sensors of bulk viscosity should be verified for hydrocolloids and for specific foods on a case by case basis.

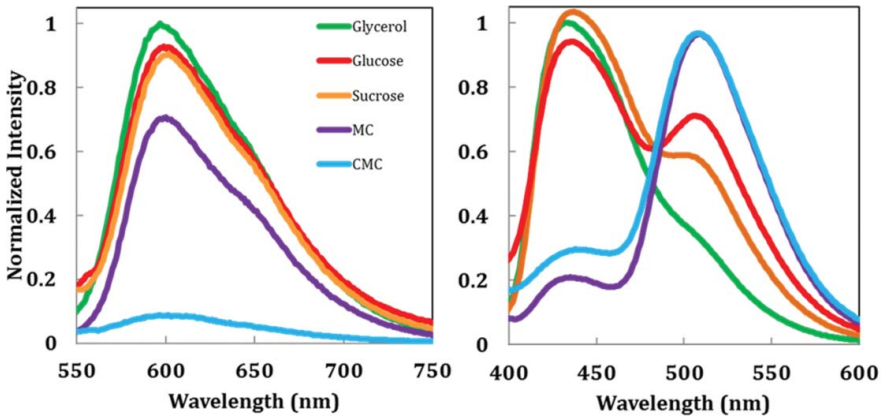


Figure 7. Normalized fluorescence intensity of the GRAS MR Azorubine (left) and pyranine (right) in solutions of different composition and equal viscosity (90 mPa s); MC: methyl cellulose, CMC: carboxymethyl cellulose. The two emission bands of pyranine are due to protonated (~435 nm peak) and unprotonated (~510 nm peak) probe. Reprinted with permission from Kashi et al. (2015).

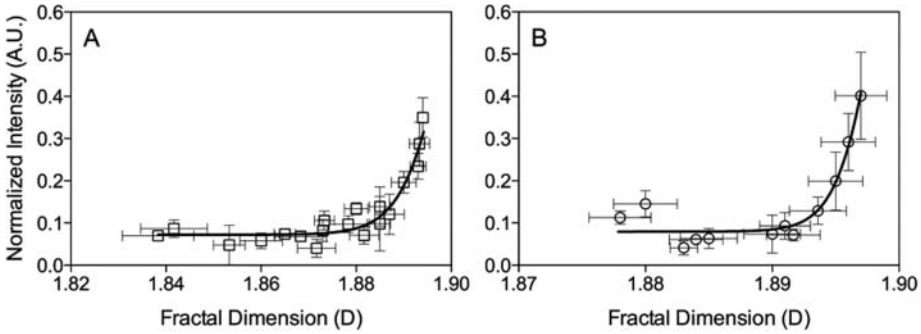


Figure 8. Normalized fluorescence intensity of Citrus Red as a function of the fractal dimension of the (A) medium chain and (B) unsaturated triglyceride crystalline networks. Reprinted with permission from Du et al. (2014).

A lipophilic azo dye, Citrus Red 2, which is also approved in food applications, was used to monitor the micro-viscosity of oil confined in colloidal fat crystal networks (Du et al., 2014). The fluorescence intensity of the dye remained constant when the degree of oil confinement, expressed in terms of the box-counting fractal dimension of the network, was below 1.89 (Fig. 8). Box-counting fractal dimension, which provides a measure of the microstructural characteristics of the crystalline network, is determined from analysis of microscopic images of the fat crystal network (Tang and Marangoni, 2006). The fluorescence intensity of the MR increased dramatically above a fractal dimension of 1.89 as the degree of confinement increased, that is, as the molecular crowding increased. In these systems, the bulk viscosity, which is dominated by the formation of a solid crystalline network, does not reflect the actual viscosity of the continuous oil phase; instead the micro-viscosity monitored by the fluorescence intensity of the embedded MR better describes the physical state of the fluid phase.

These studies provide insights and experimental opportunities applicable to the food industry. They highlight the often limited correspondence between micro and bulk viscosity found in polymer (hydrocolloid) systems while supporting the potential of MRs as non-mechanical sensors of both micro and bulk viscosity. The identification of non-toxic, non-invasive, and potentially automated non-mechanical sensors of bulk viscosity can have a significant impact on in-line measurements of physical properties of foods. In addition, they also enable completely novel applications such as measurements of viscosity *in vitro* during the process of digestion in model systems or even *in vivo* in living organisms.

A potential coupling of fluorescence spectroscopy with a dynamic gastrointestinal model such as the TNO intestinal model (TIM-1), an *in vitro* system that mimics the stomach and small intestinal compartments (duodenum, jejunum, and ileum) of the human gastrointestinal tract, is illustrated in Fig. 9. This proposed method incorporates a MR probe into the gastrointestinal solutions and uses spectrophotometry with fiber optics to measure probe fluorescence intensity during *in situ* digestion. Such an application could elucidate how postprandial macronutrient digestion, chyme viscosity, and potentially satiety are affected by initial meal viscosity, among other research questions.

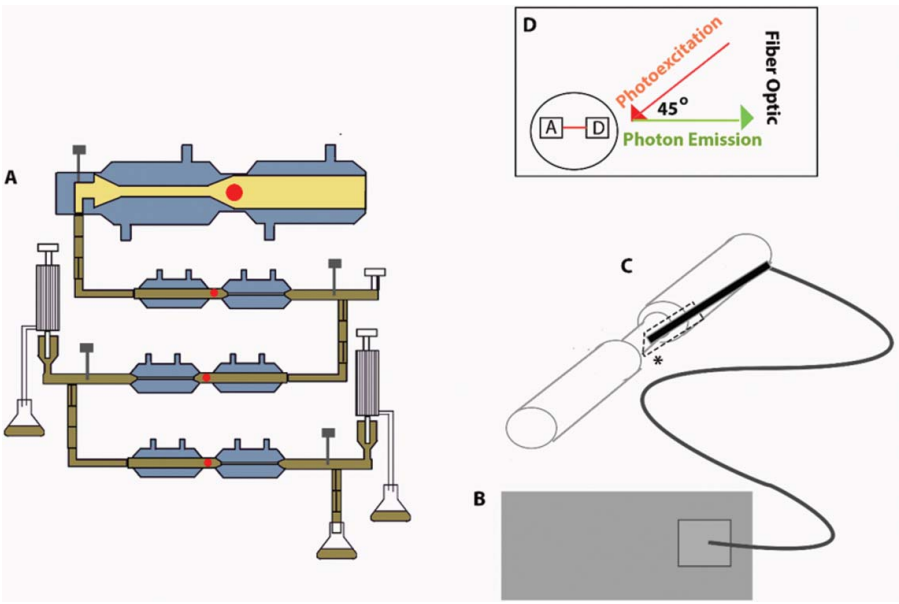


Figure 9. (A) Schematic diagram illustrating the coupling of a luminescence spectrometer with the TIM-1 gastrointestinal model system; red dots indicate locations at which (B) a fiber optic can be placed to obtain spectrofluorometric measurements. (C) Schematic diagram of the fiber optic accessory. Note the use of an adapter (dotted line) to allow repeatable positioning of the fiber optic accessory at (D) 45° angle to reduce light scattering.

Colloidal properties and dynamics

The fluorescence intensity and lifetime increases 10–100-fold compared to fluid solution when MRs are embedded in micro-organized environments such as micelles, liposomes, or inclusion complexes (e.g., cyclodextrins) (Haidekker et al., 2010; Uzhinov et al., 2011). Although there are no published reports on the use of MRs in food colloids, the advances made in a variety of non-food systems exemplify their potential to monitor physical properties and kinetics of formation of colloidal food systems.

Several MRs have been used to monitor the formation of colloidal structures. Thioflavin T shows a progressive increase in fluorescence intensity with increase in surfactant concentration up to the critical micelle concentration (Kumar et al., 2008); local environmental constraints associated with MR binding to surfactant micelles were deemed responsible for the increase in intensity. The lifetime of a synthetic MR, dubbed AzeNaph1, has been used to monitor the self-assembly of amphiphilic co-polymers into nanostructures; increased solvation of the nanostructures resulted in confinement of the micelle core as indicated by a significant increase in the lifetime of the embedded MR (Vaccaro et al., 2013). The fluorescence intensity of a GRAS probe Sunset Yellow FCF (SY; FD&C yellow #6) was used to evaluate formation of surfactant micelles (Nazar and Murtaza, 2014). Self-aggregation of SY in solution resulted in hindered rotation and an enhancement of fluorescence emission intensity; this fluorescence intensity decreased as the dye partitioned within the hydrophobic core of the micelles.

Conventional MRs, including DCVJ, CCVJ, and thioflavin T, have proved effective in following the self-assembly of dipeptide molecular gelators. The fluorescence intensity increased with fibril formation during gelation and was also sensitive to changes in structure after fibril assembly (Raeburn et al., 2015). Miguel et al. (2015) developed a MR based on the foldamer concept that was used to determine the microviscosity of an organogel formed by a urea-based organogelator and toluene. The end point of miniemulsion polymerization for an in-line process was detected using fluorescence of 1,1-dicyano-4-(4′-dimethylaminophenyl)-1,3-butadiene) (Frochot et al., 2011); although the initial polymerization steps did not affect the photophysical properties of this MR, at monomer conversions above 50%, the fluorescence intensity showed progressive increases. MRs such as merocyanine dye also have shown potential in mapping hydrophobic regions in micellar systems (Ismail, 2012).

Protein aggregation, folding, and interactions

MR binding to proteins has been shown to restrict intramolecular rotation of the probe, increasing its quantum yield, fluorescence intensity and lifetime. Further restriction of movement can be attained by conformational changes and interactions between the proteins or with other components (Uzhinov et al., 2011). The stability of proteins in parenteral nutrition products is enhanced by the addition of surfactants such as polysorbate 80, which prevents protein aggregation during the shelf life of the product. Identifying the susceptibility of these preparations to aggregation under different environmental conditions (especially variations in temperature) is crucial to ensure their adequacy and safety. The ability of CCVJ and DCVJ rotors to detect changes in aggregation in protein formulations that contain polysorbate was confirmed using steady-state and time-resolved fluorescence spectroscopy; thermally induced protein aggregation resulted in increased fluorescence intensity and longer lifetimes (Hawe et al., 2010). Differential scanning fluorimetry of CCVJ was used to detect the temperature of protein aggregation in the presence of surfactants in a high throughput screening assay (Ablinger et al., 2013). And Iio et al. (1993) used a DCVJ derivative to monitor the polymerization of G-actin into F-actin and to study F-actin filament polymorphism.

Due to the high affinity of proteins for MRs it is possible to envisioned numerous applications of MR-based fluorescence spectroscopy to monitor the structure, dynamics and functionality of food proteins including denaturation and gelation processes, stability to environmental perturbations, and interactions with proteins and other food components.

Phase transitions

Given the centrality of phase transitions in modulating the physicochemical properties of ingredients, the ability of MRs to report on phase transitions can be of significant importance to the food industry. MRs formed of polydiphenylacetylenes (PDPAs) coupled with long alkyl chains were found to be sensitive to the crystallization of paraffin (Jin et al., 2015). High rigidity of the solid state hinders intramolecular rotation and increases fluorescence intensity. Paraffins with embedded MRs were used to impregnate wax paper and develop thermo-responsive fluorescent sensor systems. ROBOD, an unconstrained boron dipyrromethene dye, was used to investigate pressure-induced changes in 1,2-dichloroethane including glass formation in the presence of an inert polymer (Alamiry et al., 2012); the high viscosity of the glassy state was found to restrict twisting of the rotor and increase fluorescence emission. Progressive vapor uptake by a polymer film results in the presumptive plasticization of the polymeric matrix; the resulting decrease in matrix rigidity was detected by a decrease in the fluorescence intensity of a julolidine-derived MR dissolved in the film (Martini et al., 2015; Minei et al., 2014). This application of MRs can be of particular importance in monitoring the stability and integrity of synthetic packaging and edible films either in the laboratory or even more importantly in the final packaged product. It should be noted that the confinement of active species such as MRs as guest within nanoporous or self-assembled materials can enhance and stabilize their functionality. Interaction of confined rotors with diffusing gaseous chemical species, make them more robust sensors of potential pollutants or vapors that can withstand temperature and pressure fluctuations (Brustolon et al., 2004; Sozzani et al., 2004)

Sensors for flow

Another relevant application of MRs, one proposed by Haidekker and Theodorakis (2007), is their use as flow sensors. Such applications provide opportunities for in-line detection of fluid

flow during continuous liquid food or juice pasteurization and in sterilization and dehydration processes where predictions of flow rates and behavior are imperative for effective processing (Holdsworth, 1971). CCVJ, a julolidine based MR, was able to report on changes in flow as a function of shear stress with a dependence on viscosity and velocity (Haidekker and Theodorakis, 2007). Mustafic et al. (2010) imaged the flow patterns in an ethylene glycol solution under four different flow chamber geometries using CCVJ and p-[(2-cyano-2-propanedio ester) vinyl] dimethylaniline (CPVDA) MRs. The images were obtained with LED illumination using a digital camera and were compared with the results of flow modeling using computational fluid dynamics. As anticipated by Haidekker and Theodorakis (2007), Mustafic et al. identified flow as the driver of the fluorescence intensity increase; however, a complete understanding of the mechanism controlling the sensitivity of MRs to flow has yet to be unraveled. Nonetheless, flow rates as low as 0.1 mm/s were detected in this work, presenting new opportunities for real-time investigations of fluid flow during food processing.

Conclusions

This review has highlighted the ability of MRs to monitor a range of physical properties of foods including bulk and micro viscosity of hydrophilic and hydrophobic liquids and semi solids, the aggregation and colloidal properties of molecules and macromolecules, the phase behavior and phase transitions of hydrophobic molecules, and fluid flow. These applications can involve measurements of bulk solutions on the macroscopic scale using a spectrometer or measurements on much smaller scales using a fluorescence microscope. MR-based fluorescence spectroscopy as an analytical technique in food systems presents distinct advantages: it is noninvasive, site specific, rapid, sensitive, versatile, and easy to use. The development of a library of edible MRs for use in food applications can enhance the use of this methodology in foods and facilitate the continuous monitoring of the physical properties of food products.

Glossary and acronyms

Bathochromic shift, red shift: A change in the wavelength of an absorption or emission band toward a longer wavelength with a lower frequency.

Fluorescence: Emission from a photoexcited singlet state where the transition from the excited state to the ground state involves emission of light with lifetimes ranging from 10^{-12} to 10^{-7} ns.

Fluorescence lifetime: The characteristic time a molecule remains in the excited state before relaxing to its ground state. Fluorescence lifetime is independent of probe concentration while fluorescence intensity is proportional to probe concentration.

Fluorescence lifetime imaging microscopy (FLIM): A technique for measuring the distribution of fluorescence lifetimes in a microscopic image. It provides information on both the spatial distribution of fluorescent molecules as well their local microenvironment.

Fluorophore: A chemical (usually aromatic) compound that can emit fluorescence upon photoexcitation; a fluorescent molecule.

Free Rotor Effect: The ability of a molecular rotor to undergo internal (segmental) rotation that is associated with non-radiative relaxation of the excited singlet state.

Hypsochromic shift, blue shift: A change in the wavelength of an absorption or emission band towards a shorter wavelength with higher frequency.

Intramolecular Charge Transfer (ICT) state: The state adopted by a molecular rotor upon relaxation of the initially photoexcited state; it involves intramolecular transfer of an electron from a donor group to an acceptor group.

Local Excited (LE) state: The planar state of a molecular rotor formed upon direct photoexcitation; it can relax directly back to the ground state with photon emission (radiative decay) or undergo charge transfer to form an ICT state.

Luminescent Molecular Rotor (MR): A molecule that consists of two (or more) segments that can rotate relative to one another (can undergo intramolecular twisting). The rate of twisting, which depends on the free volume, molecular crowding, or viscosity of the local environment, modulates the distribution of the **LE** and **TICT states**. Relaxation is primarily radiative from the LE and non-radiative from the TICT state.

Non-radiative decay rate: The rate of release of excited state energy into thermal energy (i.e., the rate of vibrational relaxation) to return to the ground state. This process does not involve photon emission and is the main mode of de-excitation of the **TICT state**.

Quantum yield: A measure of the radiative yield from the photoexcited state; equal to the ratio of the number of emitted photons to the number of absorbed photons.

Phosphorescence: Emission from a triplet state, formed by intersystem crossing from a photoexcited singlet state, where the transition from the excited state to the ground state involves emission of light with lifetimes ranging from about 10^{-4} to 10 s.

Radiative decay rate: The rate of release of excited state energy via radiative emission to return to the ground state. This process involves photon emission and occurs during de-excitation from the **LE state**.

Ratiometric sensor: A molecule (probe) engineered to consist of a segment, e.g., a luminescent molecular rotor, with specific environmental sensitivity coupled to a reference fluorophore without environmental sensitivity that is able to report on the probe concentration; the use of a ratiometric sensor can correct for the effect of probe concentration.

Resonance energy transfer (RET): Non-radiative energy transfer between two fluorophores. An excited donor (D) fluorophore may transfer energy to an acceptor (A) fluorophore through non-radiative dipole–dipole coupling. RET efficiency is inversely proportional to the sixth power of the distance between D and A and directly proportional to the spectral overlap of D emission and A absorbance.

Stokes shift: The difference in wavelength between the band maxima of the absorption and emission spectra.

Twisted Intramolecular Charge Transfer (TICT) state: The non-planar (twisted) configuration of a molecular rotor, usually with lower excited-state energy; relaxation to the ground state from the TICT state is predominantly non-radiative.

Radiative decay rate: rate of release of excited state energy via photon emission to return to the ground state. This process occurs during de-excitation from the **LE state**.

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