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Förster resonance energy transfer (FRET)-based biosensors for biological applications

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Abstract: Förster resonance energy transfer (FRET)-based biosensors have achieved great success for biological applications. However, what is not extensively appreciated is the growing role as versatile FRET biosensors within a similar biological context. This review provides a brief introduction of recent advances in principle, the designing strategies and kinds of applications of FRET biosensors. For each FRET biosensor, the appropriate background and fabrication is explained before studying their related applications. The prominent roles of nanomaterials are present in the development of more sensitive and specific FRET biosensors. Finally, the challenges and outlooks of FRET biosensors are emphasized.

Keywords: Fluorescent biosensor; FRET; Nanomaterials; Multi-FRET; Nanomedicine

1. Introduction

Fluorescent technology (Albert et al., 2000; Zhu et al., 2018; Thevenot et al., 1999)

has been extensively used in medical diagnosis (Yue et al., 2018), food safety (Xu et al., 2014), and environmental monitoring (Lu et al., 2017) because of its inherent sensitivity and high selectivity (Liu et al., 2018). However, although plenty of fluorescent biosensors have been employed for kinds of applications, they are still unsatisfactory to accurately quantitate because fluorescent signal is easily interference by many factors especially for the surrounding environment (pH and temperature) and fluorimeter (slight change of excitation or emission intensity) (Samanta et al., 2018; Mehrotra et al., 2016). To overcome the problem, the ratiometric fluorescent biosensor has been developed to quantitate the targets by the ratio of the simultaneously recording fluorescence intensity at two wavelengths (Thevenot et al., 2001; Medintz et al., 2003; Wang et al., 2018; Morris et al., 2010). The ratiometric fluorescent strategies include intra-molecular charge transfer (ICT) (Du et al., 2018; Sun et al., 2017) and FÖrster resonance energy transfer-based approach (Kikuchi et al., 2004; Feng et al., 2018; Zhao et al., 2015). For ICT, two factors usually interfere the accuracy of biosensors: 1) Binding of the targets could promote or inhibit ICT interactions to induce into remarkable shifts of the sensors absorption maxima. 2) Relatively broad fluorescence spectrum makes it difficult to accurately determine the ratio of the two fluorescence peaks. FRET can partly solve the problem because the single excitation in FRET can result in a longer emission (Zhang et al., 2008; Hu et al., 2014; Hu et al., 2013). Table 1 compares these technologies used in fluorescent biosensors.

By producing a non-invasive, real-time fluorescence signal changing with the spatial and temporal distributions of the fluorescent substance (Luby et al., 2016; Charron et al., 2018; Zhu et al., 2016), FRET has been served as the designing strategy of different biosensors for the diagnosis of disease, drug screening, and other biological applications. Besides of the high sensitivity, specificity and fast responsibility, FRET-based biosensors also have the unique advantages such as simplicity and homogeneous assay without any washing, which can be beneficial to perform accurate quantitative measurement *in vitro* and in living cells (Kikuchi et al., 2004; Zhu et al., 2016; Klymchenko, 2017; Li et al., 2017; Yuan et al., 2015; Albers et

al., 2006; Yuan et al., 2013; Chen et al., 2015).

Table 1

Comparison of commonly used fluorescence biosensors.

Development of Strategy	Disadvantages	Advantages	Ref.
Direct fluorescent biosensor	High-background	Quantitation; Semiquantitative; Cellular imaging;	(Albert et al., 2000; Yue et al., 2018; Xu et al., 2014; Lu et al., 2017)
Ratiometric fluorescent biosensor	ICT-based biosensor Serious interference	Imaging; Accurate quantitation	(Kikuchi et al., 2004; Feng et al., 2018)
FRET-based biosensor	Low-FRET efficiency	Ratiometric Imaging; Accurate quantitation	(Luby et al., 2016; Charron et al., 2018; Wang et al., 2012)

This review focuses on the biosensors relying on FRET strategies for the detection of small-molecular or biological macromolecular *in vitro*, living cells and *in vivo*. The principles of these FRET biosensors are covered, followed by kinds of applications. Moreover, we present the prominent roles of nanomaterials in the development of more sensitive and specific FRET biosensors. Finally, we also emphasized on the challenges and outlooks of FRET biosensors.

2. FRET Biosensor

2.1. Principle of FRET

FRET is an energy transfer process between a pair of light-sensitive molecules, where the donor fluorophore, initially in its electronic excited state, transfers energy to an acceptor chromophore as shown in Fig.1 (Lakowicz, 1991; Gadella, 2009; Sun et al., 2010).

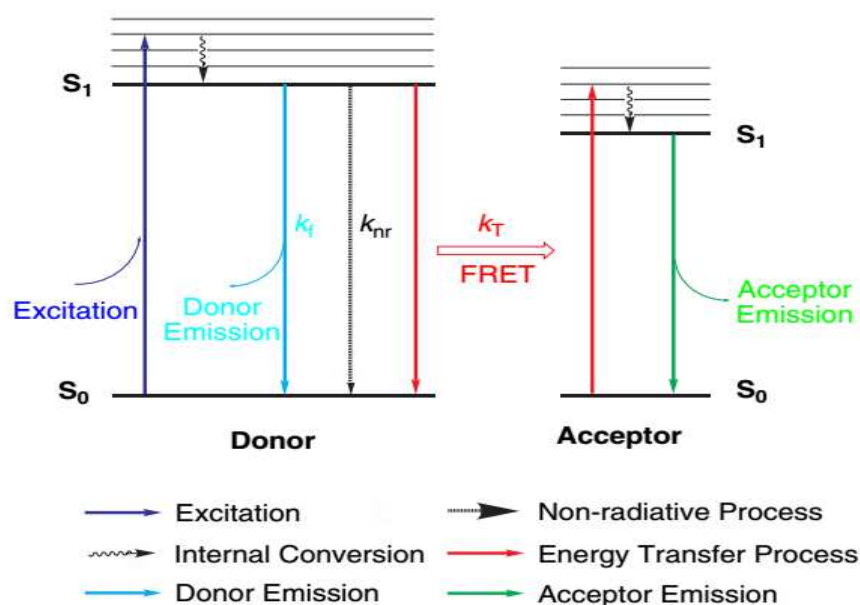


Figure 1. Basic principle of FRET. Reprinted (adapted) with permission from Kikuchi et al. (2004).

The FRET efficiency (E) depends on multiple factors that are summarized as 1) the spectral overlap (J) of the donor emission spectrum and the acceptor absorption spectrum, 2) the distance between the donor and the acceptor (typically in the range of 1-10 nm), and 3) the relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment. In the traditional FRET, one donor (D) could combine with one acceptor (A). For such the single pair of fluorophores, the efficiency (E) of FRET is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance (r) between the donor and the acceptor. Recently, a large number of biosensors based on nanomaterials have been designed. In nanomaterial-based FRET, some studies provided a theoretical model for the FRET (Berney and Danuser, 2003; Clapp et al., 2004, Corry et al., 2005; Raicu, 2007; Hildebrandt et al., 2017), which includes two cases of any As-D couple (n_a) where single donor (D) attached multiple acceptor (As), or any Ds-A couple (n_d) where single acceptor (A) can bond with multiple donors (Ds). As shown in equation (1), E can be expressed as follow by using r and R_0 .

$$E = \sum_{i=1}^{n_d} \left[\frac{R_0^6 \sum_{j=1}^{n_a} \frac{1}{r_{ij}^6}}{1 + R_0^6 \sum_{j=1}^{n_a} \frac{1}{r_{ij}^6}} \right] \frac{1}{n_d} \quad (1)$$

In Eq. (1), R_0 is the Förster distance of this pair of donor and acceptor, i.e. the distance at which the energy transfer efficiency is 50%, r_{ij} is the distance from the donor to the acceptor. Thus energy transfer was dependent on the distance between donor and acceptor.

Normally, R_0 could be 2-10 nanometers (nm) for commonly used fluorophores and relied on J and donor quantum yield (Φ):

$$R_0 = 0.0211(J\Phi k^2 n^{-4})^{1/6} \quad (2)$$

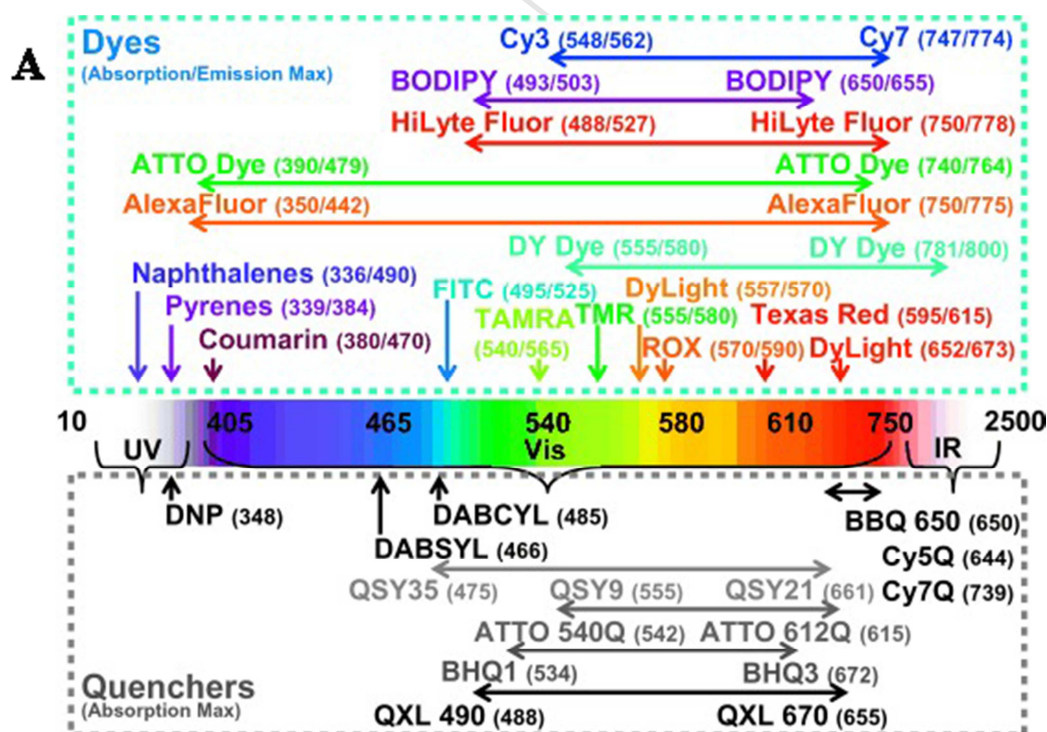
In Eq. (2), k^2 is the orientation of the donor and acceptor transition dipole moments, n is the refractive index of medium between the FRET pairs. Thus, a pair of FRET fluorophore with large spectral overlap were necessary. And the change of distance (r) between the donor and the acceptor acts as off/on the switch of FRET.

2.2. Introduction of donor and acceptor

Most commercial fluorophores can be available as FRET donors or acceptors as shown in Fig. 2. The organic donors covered ultraviolet (UV) dyes such as coumarin-, naphthalene-, and pyrene-based analogue, and near-infrared (NIR) dyes, including cyanine- (Cy3.5, Cy5.5), rhodamine. Some organic molecules were loaded or conjugated into the functionalized polymeric microspheres or onto their surface (by using functionalized groups such as biotin, avidin, amines, sulfates, and carboxylates) to increase the fluorescent signal for targets, or were modified to increase the water-solubility (Horvath et al., 2005; Zahavy et al., 2003). On the other hand, the modified fluorophores were also combined with the shell or nanoparticles (NPs) to obtain strong fluorescence (Balzani et al., 2000; Vicinelli et al., 2002; Kaafarani et al., 2003). Quenchers as the FRET acceptors are increasingly prevalent due to their wide absorption spectrum and high extinction coefficient (Maxwell et al., 2002), which included many of organics such as 4-(4'-dimethylaminophenylazo) benzoic

acid (Dabcyl) and 4-dimethylaminoazobenzene-4'-sulfonyl (Dabsyl), two of ordinary non-fluorescence acceptors, QSY9, QXL, ATTO dyes, blackberry, and black hole families (BHQ-3, BHQ-5), and metallic materials for instance gold nanoparticles due to broad absorption spectra (Sapsford et al., 2006).

Fluorescent proteins (FPs) (Fig. 2B) such as CFP, GFP, BFP and YFP have also been served as the critical players of the donor or acceptor for monitoring the protein-protein interactions or conformational changes of individual proteins in living cells (He et al., 2004; Rehm et al., 2002; Periasamy, 2001; Enterina et al., 2015; Kyrychenko et al., 2017). Besides, lanthanides cations such as terbium and europium (Fig. 2D) as long-lifetime dyes, have emerged as the prominent role of time-resolved FRET system (Selvin, 2005; Pulli et al., 2002; Qin et al., 2003). Moreover, fluorescent nanoparticles including quantum dots (QDs) (Fig. 2C) and carbon dots (CDs) with broad absorption, can serve as both donor and acceptor (Sidhu et al., 2017; Russ Algar et al., 2011). The structure of linker space and property of fluorophore pair could also affect the intrinsic FRET efficiency of the probe (Mccartney et al., 2017).



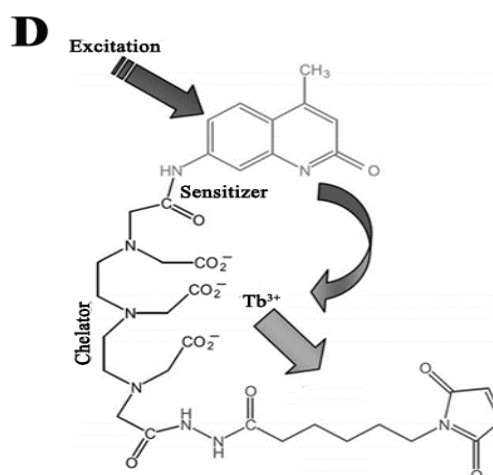
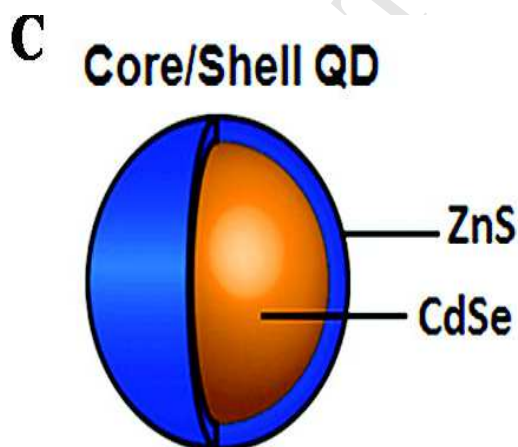
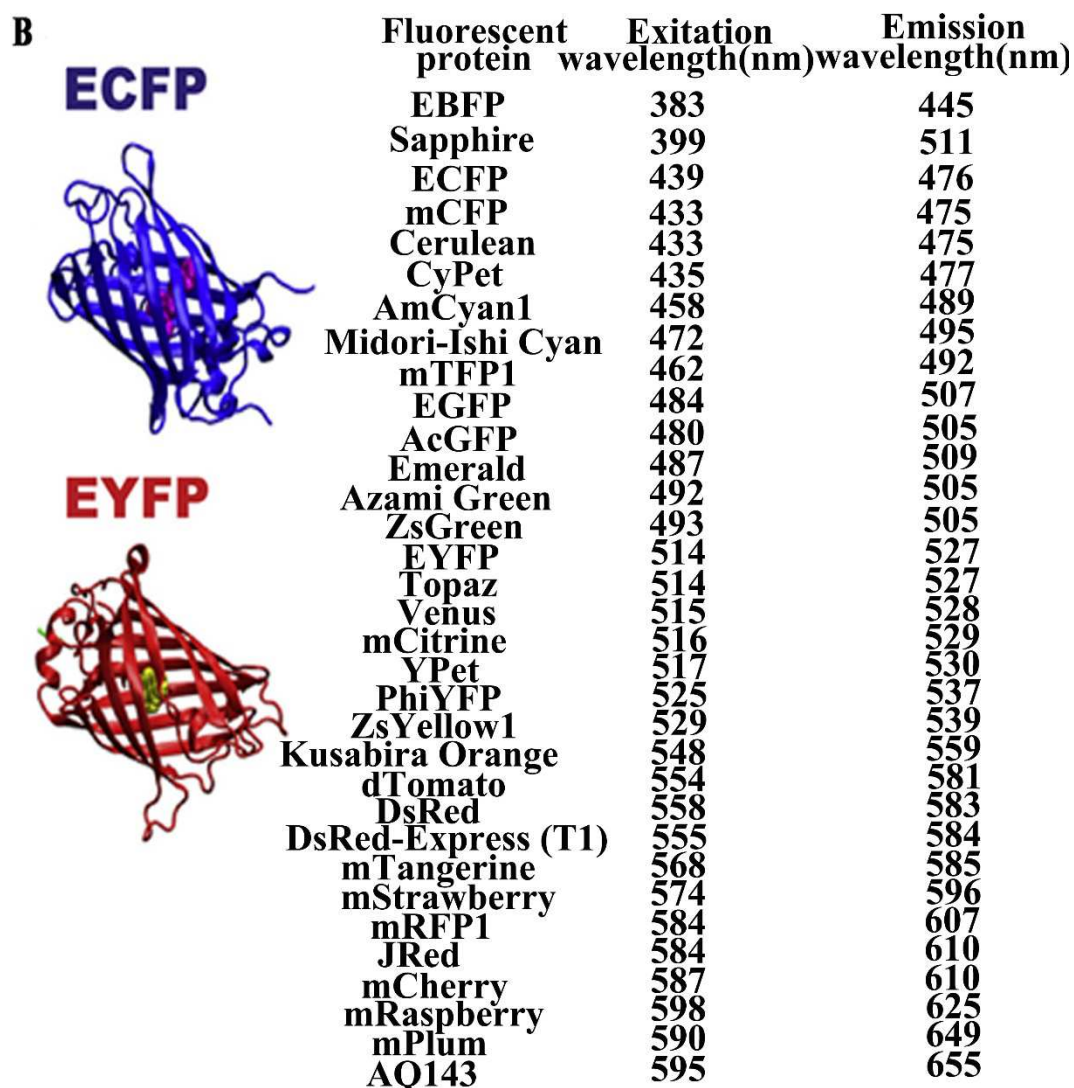


Figure 2. (A) Absorbance and emission maxima of common available fluorescent dye and quencher families. Reprinted (adapted) with permission from Sapsford et al. (2006). (B) Simplified structure of fluorescent proteins, ECFP and FYFP and lists of commonly used fluorescent proteins. Reprinted (adapted) with permission from

1 Kyrychenko et al. (2017). (C) Cartoon structure of a CdSe/ZnS QD. Reprinted
 2 (adapted) with permission from Russ Algar et al. (2011). (D) Structure of the Tb probe.
 3 Reprinted (adapted) with permission from Sapsford et al. (2006).

4 2.3. Fabrication of FRET-based biosensor

5 Multiple kinds of FRET biosensors are designed as shown in Table 2. Commonly
 6 used FRET biosensor was fabricated by a pair of donor and acceptor with a close
 7 position, and a specific receptor (such as protein, DNA) was used to identify the target.
 8 The structure of the biosensor is sandwiched between the donor and the acceptor
 9 (Peroza et al., 2015; Mohsin et al., 2014). After binding of receptor with the targets,
 10 FRET signal is observed due to the destruction of the pair of donor and acceptor
 11 because of the conformation change or the cleavage of the receptor. It has been
 12 utilized for quantifying analysis of targets such as ions, proteins and nucleic acid *in*
 13 *vitro* or in living cells (Klymchenko, 2017; Li et al., 2015). For whole-animal imaging
 14 by *in vivo* microscopy, many FRET biosensors were limited due to the attenuation of
 15 the fluorescence and absorption of the tissue for visible light (Leeuwen et al., 2015).
 16 And the ideal fluorophores for animal imaging should have emissions at the NIR
 17 region (>700nm), where the absorption, light-attenuation, and auto-fluorescence are
 18 minimum (Guo et al., 2013; Luo et al., 2011; Kobayashi et al., 2010).

19 Recently, a dual-FRET biosensor was successfully fabricated for the sequential
 20 sensing of MMP-2 and caspase-3 via a dual-stage turn-on fluorescence processes (Li
 21 et al., 2015). The biosensor was comprised of 5(6)-carboxyfluorescein (FAM) as
 22 donor and two 4-{[4-(dimethylamino)-phenyl]-azo}-benzoic acid (Dabcyl) moieties
 23 as FRET quench fluorophore pairs. While, MMP-2 specific peptide sequence and
 24 caspase-3 sensitive peptide unit were utilized as cleavable substrates between the
 25 dual-FRET pairs, respectively. In addition, a multi-step FRET biosensor was used to
 26 study molecular interaction beyond 1-10 nm through the energy transfer by an FRET
 27 antenna (Saha et al., 2016), and which would expand the application of FRET
 28 biosensor in biology research. In this biosensor, energy transfer from pyrene (Py) to
 29 rhodamine B (RhB) occurred in two steps-(i) firstly from Py to Acriflavine (Acf) and

(ii) secondly Acf transfer this energy to RhB. Also direct energy transfer from Py to RhB may become significant in presence of Acf.

On the other hand, some peculiar nanostructures such as mesoporous silica nanoparticles (MSNs), liposomes, polymer micelles, scaffolds and nanoemulsions were also utilized as FRET-based biosensors for signal enhancement due to their low toxicity and the improvement of energy transfer efficiency (Bremer et al., 2001; Torchilin et al., 2005; Webster, 2006; Park et al., 2009; Yan et al., 2013; Li et al., 2013; Wang et al., 2016; Pelaz et al., 2017; Lane et al., 20015). For example, MSNs possess excellent capacity for cargo loading due to stable mesoporous structures, large surface area, tunable pore sizes, and well-defined surfaces. The fluorophores of FRET pairs were simultaneously encapsulated in the inner or combined onto the surface of MSN to increase the fluorescence signal, obtain lower detection limits, and preserve the function of the targets (Charron and Zheng, 2018; Thapaliya et al., 2015). Other various integrated FRET biosensors have also been achieved for the applications in real-time imaging, theranostics and sensing with a non-invasive way in living cells or animal (Kunjachan et al., 2015; Toy et al., 2014; Smith and Gambhir, 2017; Petersen et al., 2012; Mura et al., 2012; Zheng et al., 2015; Charron et al., 2015; Mirkin et al., 2015; Chen et al., 2017; Chen et al., 2016; Ekdawi et al., 2015). By using these FRET biosensors, the micro-distribution and subcellular localisation of nanosensor in ex vivo tissue sections is also becoming feasible (Ekdawi et al., 2015). These FRET biosensors provide the advantages of tracking nanomedicine from subcellular to whole-animal due to its sensitivity and accessibility (James and Gambhir, 2012; Priem et al., 2015; Pansare et al., 2012; Yao et al., 2014; Merian et al., 2012).

Table 2
Multiple FRET-based biosensors.

Description	Measuring parameter	Feature	Application	Ref.
Traditional FRET biosensor		a pair of donor and acceptor with the distance of 1-10 nm	Detection of the conformation; ions, proteins and nucleic acid <i>in vitro</i> and in living cells	(Peroza et al., 2015; Mohsin and Ahmad, 2014)

dual-FRET biosensor	Ratio of fluorescent intensity	a fluorophore and two quenchers with the distance of 1-10 nm	one on-going pathology	(Li et al., 2015; Leeuwen et al., 2015)
multi-step FRET sensor		molecular effect beyond 1-10 nm	research among molecular interaction	(Saha et al., 2016)
peculiar nanostructures for FRET sensor with signal enhancement		A nanocarrier system	tracking drug from subcellular to whole-animal; a non-invasive ways	(Toy et al., 2014; Smith and Gambhir et al., 2017; Petersen et al., 2012)

2.4. Analysis of FRET data

FRET is usually detected by appropriately equipped fluorescence spectrometer or microscopes, either as a decrease in the fluorescence intensity, lifetime, or anisotropy of the donor, or as an increase in the fluorescence intensity of the acceptor upon donor excitation (if the acceptor is fluorescent), or as a combination of these two effects. A FRET ratio of A/D was introduced as a variable in the FRET signal that corresponds to the amount of targets, where A and D respectively represent the maximal fluorescent intensity of the acceptor and donor (Zhou et al., 2012; Torchilin, 2014). The crosstalk of spectrum between the donor and acceptor is a major challenge for quantitative FRET analysis. The donor emission can overlap with the acceptor emission, and the acceptor may also be directly excited at the donor excitation wavelength. Multiple efforts have been attempted to overcome these challenges. Fluorophores exhibiting a large Stokes shift, such as quantum dots and upconversion nanocrystals, have been used to reduce the crosstalk. Moreover, time-resolved FRET assays with fluorophores that exhibit long half-lives, such as lanthanide chelate dyes, have proven effective in eliminating interfering fluorescence. Liao group developed a method to mathematically analyze the spectrum crosstalk between the FRET donor and acceptor by introducing a second excitation (Liao et al., 2015). In this assay, an emission wavelength at which only the donor emits is used to calculate donor emission at the acceptor emission wavelength, and a second excitation wavelength at which only the acceptor emits is used to calculate the acceptor direction emission. Jin

et al provides a unique combination of correction performance parameters by careful implementation of several spectroscopy technologies (time-resolved fluorescence detection, color multiplexing, biospectral crosstalk correction) into a FRET nucleic acid assay (Jin et al., 2015). Chen et al also developed a correct approach to quantitatively analyze the FRET emission (Chen et al., 2007). Three filter tubes are utilized to collect fluorescence images of the donor emission at the donor excitation, the acceptor emission at the donor excitation, and the acceptor emission at the acceptor excitation. These collected signal intensities are then expressed as functions of four crosstalk parameters and three fluorescence components: donor fluorescence, sensitized acceptor FRET emission, and the direct emission of acceptor. The crosstalk parameters were determined from samples with only donor or acceptor, and the three fluorescence components were calculated by a three-variable linear equation group. Although these approaches greatly reduce spectrum crosstalk in the FRET assay and simplify FRET signal analysis, more generalized approaches are needed.

The fluorescent imaging based on FRET can give spatial data and optical information (Ray et al., 2014). FRET imaging could also obtain abundant visual information in regard to nanoparticle integrity, efficiency and behavior between the biosensors with proteins, or living cells. For FRET imaging, the semi-quantitative result was achieved by analyzing the ratio of corresponding pixel intensity at donor and acceptor channels *in cell* and *in vivo* (Zhang et al., 2018; Bouchaala et al., 2016).

3. Application of FRET-based biosensor

FRET plays significant role in designing various biosensors for the clinical, pharmaceutical, toxicological, or agri-food analysis among others (Jin et al., 2010; Xu et al., 2017; Limsaku et al., 2018; Ibraheem and Campbell, 2010; Tian et al., 2010; Saha et al., 2018; Barker et al., 1998). According to the analytes (in Table 3), the FRET biosensors can be classified including small molecules, antibodies (immune molecules), enzymes, nucleic acids, and tissue-based biosensors (Mehrotra, 2016; Mohsin et al., 2015; Goldsmith, 1975). Recently, FRET biosensors have also been successfully utilized for the detection of compounds, *in vivo* imaging, drug delivery,

1 and image-guided surgery (Shen et al., 2014; Rai et al., 2015; Zhang et al., 2006).

2 **Table 3**

3 Summary of recent applications of FRET-based biosensor.

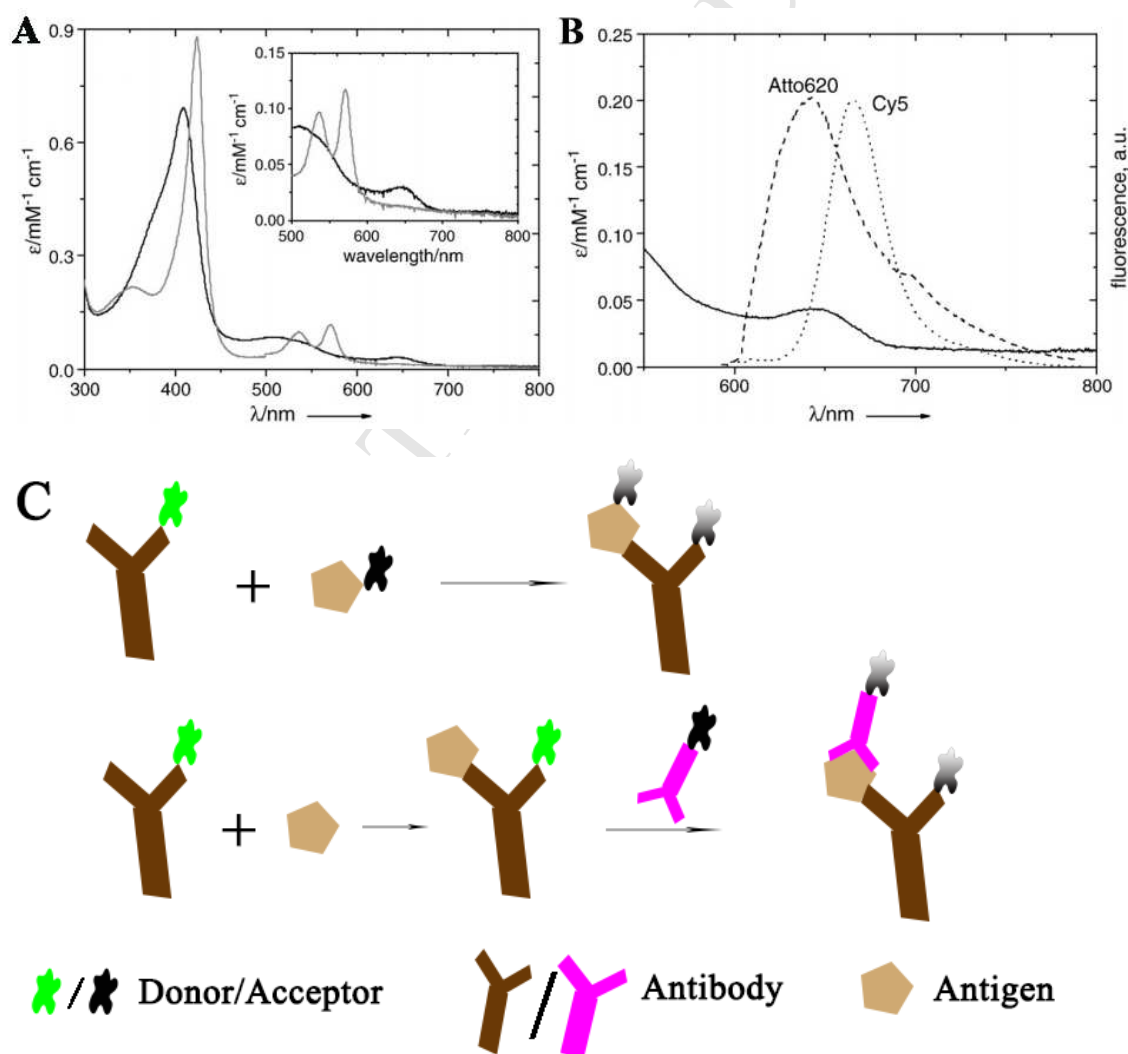
Analyte	Fluorophore	Receptor-Linker	Ways	LOD/linear range	Ref.
Small molecules					
NO	Atto620/Cy5	cytochrome c peroxidase	Content	10 nM	(Strianese et al., 2010)
Zinc	CFP/YFP	Metallothioneins SmtA	Content	0.1 μ M	(Mohsin et al., 2015)
Immune molecules					
aflatoxin B ₁	Red/Green emission QDs	Monoclonal anti-AFB ₁	Content	0.1302 pM	(Xu et al 2014)
Enzymes					
neutrophil elastase	YFP/CFP	a specific recognition sequence	Activity	na	(Faccio and Salentinig, 2017)
matrix metalloproteinase 9	5-FAM/QXL520	specific peptide substrate	Activity	na	(Lee et al., 2018)
BSA/HSA	pyrimidine derivative	Schiff-base ligand	Conformational change of protein	na	(Ghosh et al., 2018)
calpain	CFP/YFP	calpain cleavage sequence	Activity	na	(Mccartny et al., 2017)
MMP-2/caspase-3	FAM/Dabcyl	cleavable substrates of MMP-2 and caspase-3	Activity	Tumours ~1cm in diameter	(Kwon et al., 2017)
MMP-2/MMP-7	AMC/BCTOT-Eu ^{II}	cleavable peptide spacer	Activity	na	(Wang et al., 2012)
MT1 MMP	ECFP/R-PE	PEbody	enzymatic activity at the surface	na	(Limsakul et al., 2018)
telomerase	fluorescein /BHQ	Loading with MSN	activity	na	(Qian et al., 2013)

Nucleic acids					
DNA	Atto 488/ Atto 647N	dye-labeled “imager”/complementary strands		~72 nM	(Auer et al., 2017)
ochratoxin A	colloidal cerium oxide nanoparticles/G QDs	OTA aptamer (electrostatic assembly)	Content	2.5 pg mL ⁻¹	(Tian et al., 2017)
SYBR Green I	anthanide-doped NPs(UCNPs)/ss DNAs	coordination interaction		3.2 nM	(Wu et al., 2018)
Tissues					
organs	Cy 5.5 / Cy 7.5	encapsulation	Content	na	(Bouchaal a et al., 2016)
Thermal-responsives					
	FITC/Rhodamine B (RB)	PECT self-assembly	imaging	na	(Huang et al., 2017)

1 3.1. Small molecule FRET biosensors

2 FRET biosensors have been utilized for detection of many small molecules such
3 as nitric oxide (NO), metal ions and other organic small molecules (Tian et al., 2010;
4 Saha et al., 2018). NO plays an important role in multitudinous biological functions
5 such as the carcinogenic and neurodegenerative disorders (Culotta and Koshland,
6 1992). A fast and reversible fiber-optic NO-selective fluorescent biosensor was
7 successfully developed with a detection limit of 20 μ M and linear up to 1mM NO by
8 immobilizing the Cytochrome C at end of optical fiber in poly-acrylamide-matrix or
9 covalent binding to the gold nanobeads (Barker et al., 1998). After that, Maria
10 Strianese designed a NO-sensitive FRET sensor, where cytochrome c peroxidase (CcP
11 Ex= 645 nm) was labeled by a fluorophore (Em= 645 nm) as shown in Figure 3A. A
12 FRET was occurred by the energy transfer from the labeled fluorophore (Atto620 or
13 Cy5) to the protein NO-free labeled cytochrome c peroxidase (CcP) (Strianese et al.,
14 2010). At the presence of NO, the CcP exhibited a disappearance of absorption
15 spectrum at 645 nm ascribed to the conformational changes of CcP after the binding

with NO, resulting in the renewing of the fluorescence. The biosensor showed a lower detection limit of 10 nM compared to the previous work (Barker et al., 1998), demonstrating FRET biosensor can be used for the detection of extremely low NO level. Other than NO, Mohd. Mohsin reported a fluorescent protein-based biosensor for the detection of Zinc *in vitro*, in living cells and microorganisms (Mohsin et al., 2015). In the FRET pair, CFP (Em 485 nm) and YFP (Em 535 nm), was covalently linked to the two flank, N- and C- terminus of signal peptide of Metallothioneins SmtA, a cysteine-rich protein in *Cyanobacterium Synechococcus* sp. The FRET signal was increased significantly with the increase of zinc concentration, providing a linear detection range for Zn quantification between 5.0 μ M and 2.0 mM, which is consistent with previous report (Duncan et al., 2006).



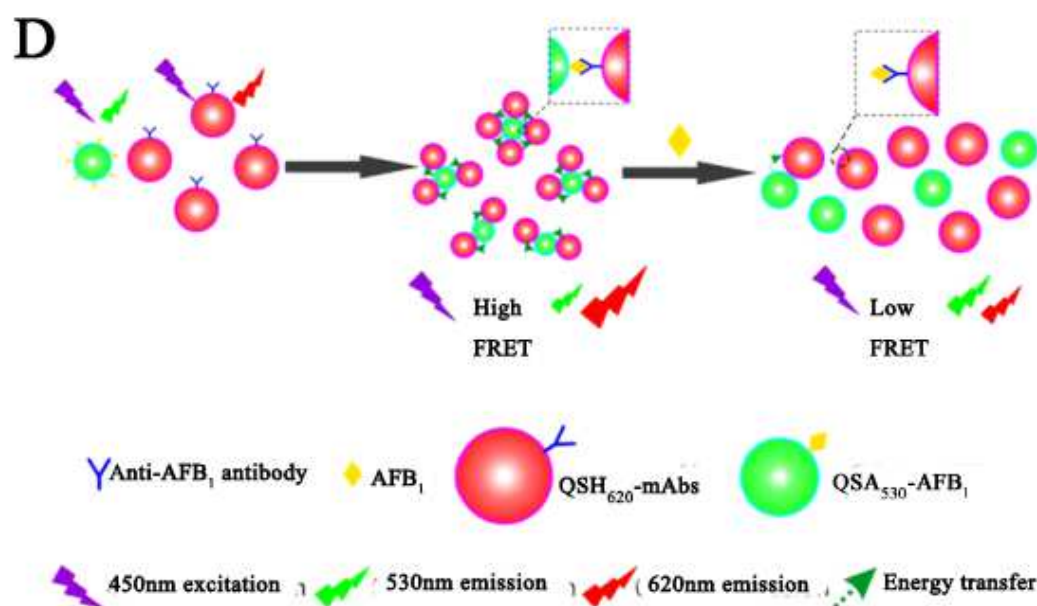


Figure 3. (A) Absorption spectrum of NO-free (black) and NO-bound (grey) CcP. (B) Absorption spectrum of NO-free CcP (solid line) and emission spectra of Cy 5 (dotted line) and Atto 620 (dashed line). Reprinted (adapted) with permission from Strianese et al. (2010). (C) Commonly used FRET-based biosensor consisted of antibody and antigen. (D) Schematic structure of FRET-based immunosensor for the sensitive detection of AFB1. Reprinted (adapted) with permission from Xu et al. (2014).

3.2.FRET Immunosensors

In addition to small molecules, FRET biosensors have also been applied for the detection of immune molecules (Goldsmith, 1975; Haab, 2003; Zhu et al., 2010; Lai et al., 2013; Zhu et al., 2014; Zhu et al., 2015; Lequin, 2005). And the FRET biosensors were mostly linked via $-\text{SiOCN}/-\text{NH}_2$, $-\text{COOH}/-\text{NH}_2$, and biotin/streptavidin (Kumar et al., 2009; Wang et al., 2014; Wang et al., 2014; Song et al., 2012; Zhang et al., 2011). Their fabrications are various as demonstrated in Figure 3C. Compared to standard enzyme-links or fluorescent sandwich immunoassays, FRET-based immunosensors have unique merits of free operation of washing and separation steps, excellent stability, specificity and ratiometric measurement (Hildebrandt et al., 2017; Haab et al., 2003). A great amount of FRET-based immunoassays had been successfully developed (Xu et al., 2014; Knopp, 2006).

Kattke et al. designed a FRET biosensor for the detection of mold spores in solution (Kattke et al., 2011). They conjugated fluorescent QDs to antibodies, which were then mixed with a solution of spores labeled with a quencher. The fluorescence was recovered upon addition of unlabeled spores in a dose-dependent manner, owing to the displacement of the QD-antibody conjugates from the quencher-spores. This FRET biosensor enabled detection of spores doses as low as 10^3 spores/mL. Stinger et al. proposed a FRET biosensor for the detection of antigen based on the conformational changes upon binding to the antigen (Stringer et al., 2008). They first conjugated protein A onto the surface of QDs, which enabled the oriented assembly of a dye-labeled IgG antibody against the Troponin I protein, resulting in FRET between the QD and dyes on the antibody. The assay could obtain a limit of detection (LOD) of the 200 nM. Xu reported a FRET-based immunosensor for the sensitive detection of aflatoxin B₁ (AFB₁) with a LOD of 0.13 pM (0.04 ng/ml) in rice grains using different-sized QDs (Xu et al., 2014). As shown in Figure 3D, to avoid potential irregular aggregation between two kinds of QDs, monovalent monoclonal antibody (mAb)-labeled red QDs (~0.84 anti-AFB₁ mAbs per QD) and multivalent hapten-labeled green QDs (~6.8 AFB₁ per QD) were utilized as acceptor and donor, respectively. The interactions of anti-AFB₁ mAbs and AFB₁ promoted one or more acceptors bound with a multivalent AFB₁-labeled donor, resulting in energy transfer from the green QDs to the red QDs. This sensor performed a linearity with concentration range of 0.19-16 pM, indicating the FRET can be a promising method for AFB₁ in food safety at a low concentration (Xu et al., 2014).

3.3. Enzymatic FRET biosensors

The FRET biosensors are expansively exploited for the detection of biomarkers and enzymatic activities *in vitro* and in/on the surface of living cells (Zadran et al., 2012; Cao et al., 2007; Lee et al., 2018). Commonly, the FRET biosensors were designed for the detection of proteins dependent upon two elements, a pair of donor-acceptor and a specific recognition module for the targets (Fommer et al., 2009; Mertens et al., 2012; Kikhney et al., 2015). A conformational change of the targets

1 results in the switch of “turn on/off” of FRET. Greta Faccio et al. fabricated a
2 FRET-based biosensor for the detection of neutrophil elastase (NE) activity (Faccio et
3 al., 2017). As shown in Figure 4A, YFP and CFP was covalently combined with the
4 linker, which has a specific recognition sequence for NE that belongs to a relevance
5 inflammatory serine protease. Ghosh also designed a biosensor based on FRET by
6 utilizing the synthesized pyrimidine derivative as fluorescent probe in Figure 4B
7 (Ghosh et al., 2018). A kind of pyrimidine based Schiff-base ligand,
8 2-(2-(Anthracen-9-ylmethylene) hydrazinyl)-4,6-dimethyl pyrimidine (ANHP), was
9 used to ascertain the conformational change of protein, and the FRET biosensor
10 showed high sensitivity.

11 Sidhu designed a highly selective biotin-CDs-naphthalimide biosensor for
12 monitoring the activity of thioredoxin reductase (TrxR) in cancer cell and
13 pharmacological screening of cancer (Sidhu et al., 2017). As shown in Figure 4C, a
14 FRET platform was prepared by conjugating naphthalimide onto the surface of CDs
15 through a disulfide linkage, which obtained a limit of detection of 7.2×10^{-8} M TrxR.
16 Similarly, Lee fabricated an enzymatic-sensitive inkjet printing FRET biosensor for
17 high-throughput screening assay against MMP-9 using 5-FAM as a fluorescent donor
18 and QXL520 as a quencher acceptor in Figure 4D (Lee et al., 2018). This method
19 holds remarkable merits in extensive applications for the measurement of MMP
20 enzyme family and rapid evaluation of anticancer compounds. And Qian described a
21 switchable FRET system based on telomerase-responsive MSN to realize in situ
22 “off-on” imaging of intracellular telomerase activity (Figure 5A) (Qian et al., 2013).
23 After loading fluorescein into the mesopores, MSN was wrapped by DNA (O1) to
24 obtain a telomerase-responsive biosensor. In the presence of telomerase, the biosensor
25 could generate fluorescence because of the detachment of O1 on the surface of the
26 MSN.

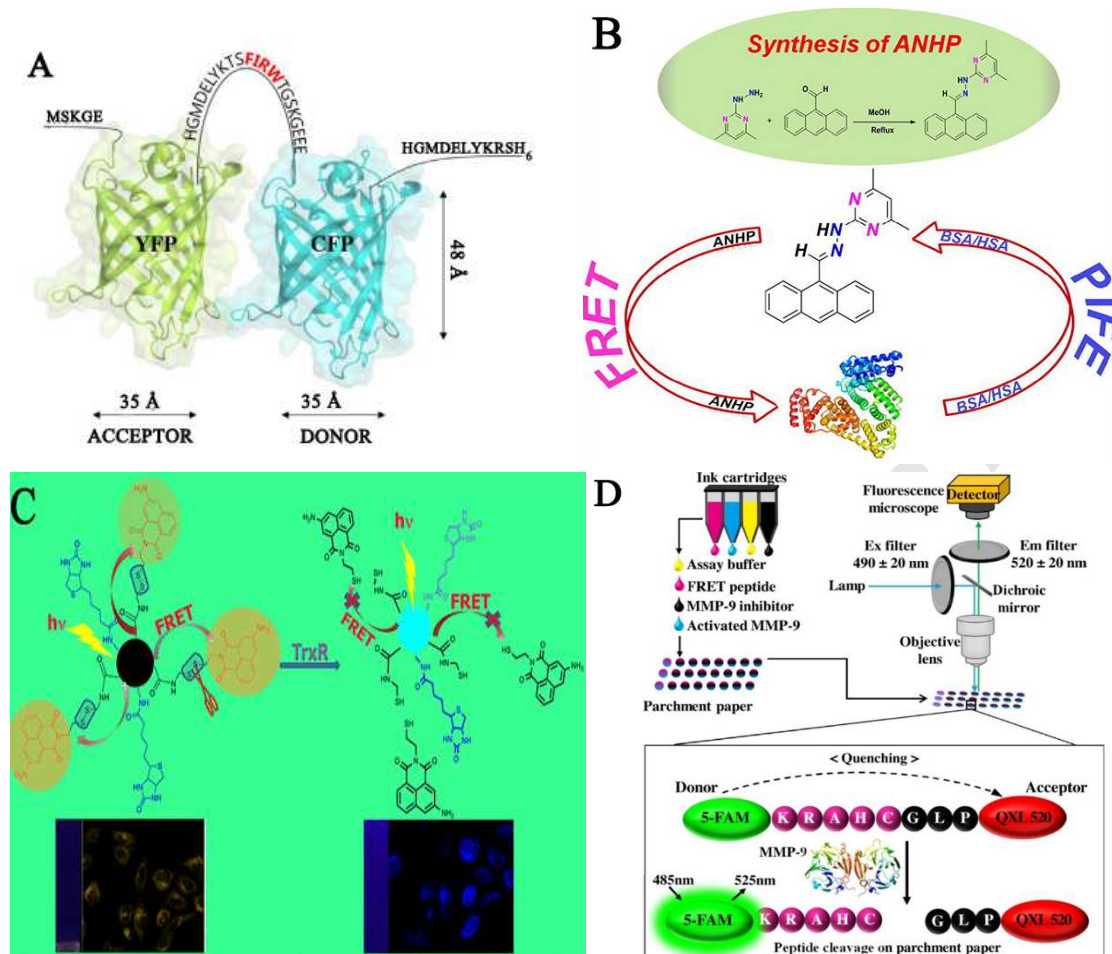
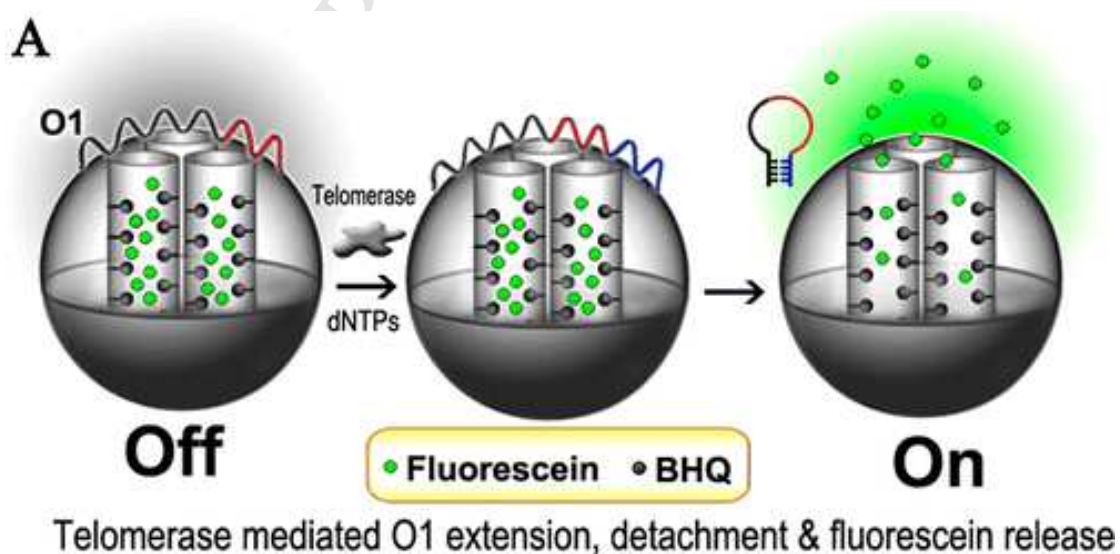


Figure 4. (A) Schematic illustration of FRET-based biosensors based on a fluorophore protein pairs for the detection of NE activity. Reprinted (adapted) with permission from Faccio and Salentinig. (2017). (B) FRET-based biosensor for the detection of protein conformational change. Reprinted (adapted) with permission from Ghosh et al. (2018). (C) A highly selectively FRET biosensor for monitoring of the activity of TrxR. Reprinted (adapted) with permission from Sidhu et al. (2017). (D) Diagram of biosensor based on a quencher and fluorophore for the target of MMP-9 in anticancer screening assay. Reprinted (adapted) with permission from Lee et al. (2018).

Several researches have also fabricated FRET biosensors for the detection of proteases collagenase (MMP-9, MMP-2), thioredoxin reductase, caspase-3 and others zymoproteins in living cells (Shi et al., 2006; Ren et al., 2014; Kwon et al., 2017). These enzymes in pathological state are over-expressed, in which case the biosensors in the surrounding medium may respond adequately. Xu designed a dual-labeled

FRET-based biosensor for the simultaneous imaging of MMP-2 and MMP-7 of living cells under single wavelength excitation as shown in Figure 5B (Wang et al., 2012). MMP-sensitive biosensor was prepared by bio-conjugating a double-labeled fluorescent cleavable peptide spacer, where 7-amino-4-methylcoumarin (AMC) and 1,10-bis(5'-chlorosulfo-thiophene-2'-yl) -4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (BCTOT-Eu^{III}) was respectively combined to the N terminus and the C terminus of two substrate peptides (a MMP-2 substrate and a MMP-7 substrate), onto the surface of Au NPs through cysteine residues of peptide substrate. At the presence of the complexes of MMP-2 and MMP-7, the FRET process of biosensor was destructed, and the fluorescence of dye was recovered at 449 nm and 613 nm. The strategy exhibits high specificity against BSA and normal-cell for simultaneous imaging of dual components of MMP-2 and MMP-7 in a complex system, and the biosensor can effectively avoid negative outcomes in cancer cell diagnosis (Wang et al., 2012). Li also designed a dual-FRET biosensor to target MMP-2 and caspase-3 for screening of cancer drug and therapeutic effect evaluation as shown in Figure 5C (Li et al., 2015). The FRET system is comprised of quencher-fluorophore pairs, a 5(6)-carboxyfluorescein (FAM) (donor) and two Dabcyl molecules (accepter), and the cleavable substrates containing MMP-2 specific peptide unit and caspase-3 sensitive peptide



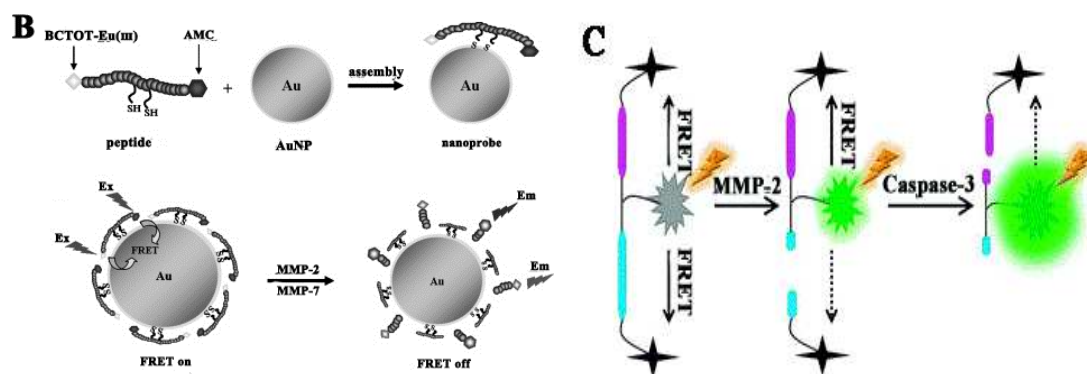


Figure 5. (A) Schematic illustration of FRET nanocarrier based on MSN for living cell imaging. Reprinted (adapted) with permission from Qian et al. (2013). (B) A dual-FRET biosensor for detecting MMP-2 and MMP-7. Reprinted (adapted) with permission from Wang et al. (2012). (C) Structure of dual-FRET biosensor for two targeted analytes, MMP-2 and caspase-3. Reprinted (adapted) with permission from Li et al. (2015).

And the improvement of monitoring the enzymatic activity at the surface of living cells using a FRET-based biosensor was also achieved by Limsakul (Limsakul et al., 2018). They reported a hybrid biosensor for *in situ* monitoring the membrane type 1 matrix metalloproteinase (MT1 MMP) activity. The successfully integrating of monobody variant (PEbody, a specific binding partner for R-phycoerythrin (R-PE)) into a FRET biosensor was capable to monitor cell-cell junction maturity for the dynamic formation and dissociation of cell-cell contacts.

3.4. Nucleic acid-based FRET biosensor

DNA or RNA molecules, such as aptamers, could specifically combine with the complementary targets by typical Watson-Crick based-pairing, or bind with the targets including small molecules, proteins and larger cells by specific folding three-dimensional conformations (Famulok et al., 2007; Ma et al., 2015). Specific combination exhibited huge merits such as high affinity, selectivity, stability, low cost and high thermal stability as shown in Table 3 (Chen et al., 2014; Yuan et al., 2014). Most hybridization assays of nucleic acid-sensing FRET biosensors were achieved by the sandwich, displacement and non-specific assemblies (Auer et al., 2017; Tian et al.,

2017; Wu et al., 2018; Climent et al., 2010).

Alexander Auer reported a fast, background-free FRET biosensor for DNA point accumulation in nanoscale topography (DNA-PAINT) imaging by the predictable and transient hybridization between short “imager” labeled by dyes and complementary target-bound “docking” strands (Figure 6A) (Auer et al., 2017). This biosensor could achieve high-quality and super-resolution imaging of DNA for dynamic process in cellular environment in few tens of seconds. Jepsen developed a FRET biosensor, in which using fluorescent RNA aptamers to produce a dynamic and reversible RNA nanodevice for tracking RNA molecules in *E. coli* (Jepsen et al., 2018).

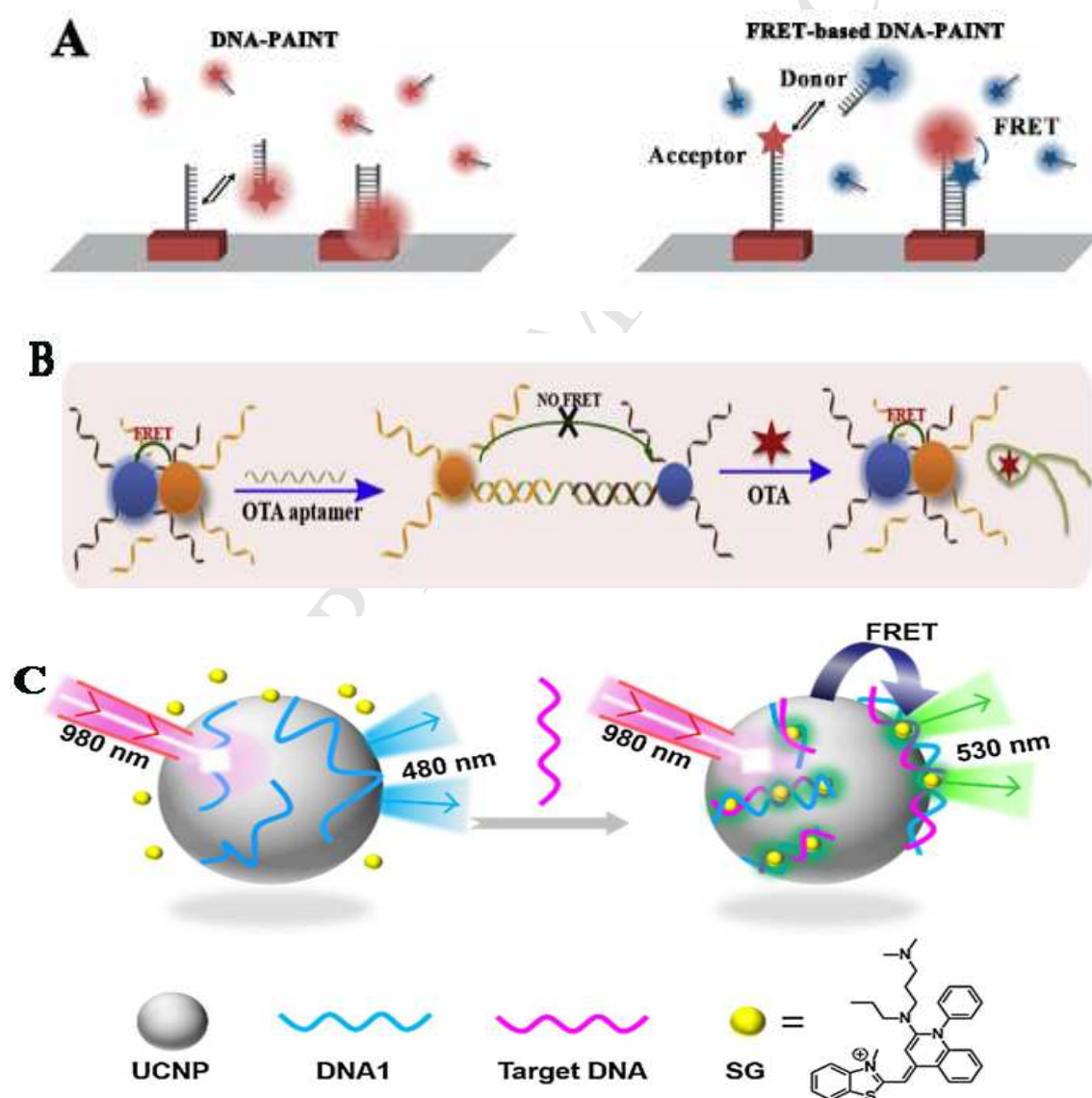
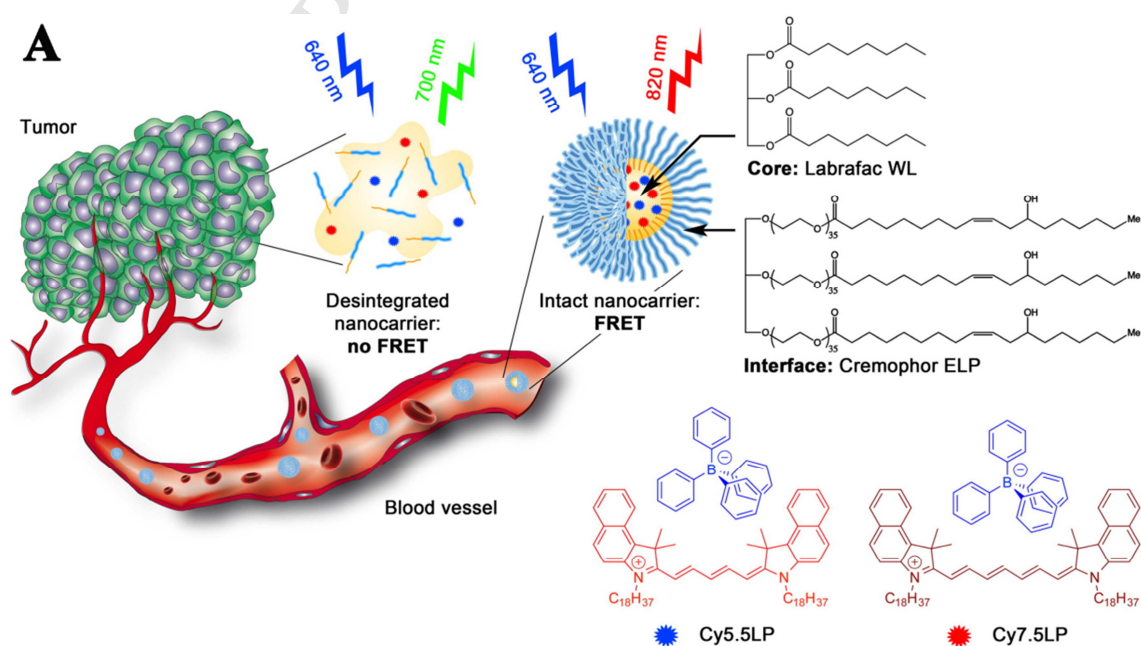


Figure 6. (A) Comparison diagram of FRET-based and conventional probe for DNA-PAINT imaging by hybridization. Reprinted (adapted) with permission from

Auer et al. (2017). (B) A ratiometric aptasensor for the detection of OTA in feed and food. Reprinted (adapted) with permission from Tian et al. (2017). (C) Schematics of near-infrared responsive FRET-based DNA biosensor based on UCNPs and SG for exploring the optimal nanoparticle size. Reprinted (adapted) with permission from Wu et al. (2018).

A FRET ratiometric aptasensor was designed for the detection of ochratoxin A (OTA) in feed and food chains by using colloidal cerium oxide nanoparticles (the donor) and graphene quantum dots (GQDs, the acceptor) (Tian et al., 2017). In this biosensor, DNA@nanoceria and DNA@GQD was designed to complement with OTA aptamer by the electrostatic force as shown in Figure 6B. The aptasensor exhibited a satisfying linear range ($0.01\text{--}20\text{ ng} \cdot \text{mL}^{-1}$), a low limit of detection ($2.5\text{ pg} \cdot \text{mL}^{-1}$), and high selectivity towards OTA. Wu et al designed a highly efficient FRET biosensor for DNA hybridization detection by using small upconversion nanoparticles (UCNPs) and nucleic acid stain SYBR Green I (SG, a specific intercalator of double-stranded DNA) as shown in Figure 6C (Wu et al., 2018). The 10-nm-UCNP-based DNA biosensor could reach a lower detection limit of complementary ssDNA at 3.2 nM and three-base mismatched ssDNA2-M3 at 7.6 nM . Xu also demonstrated a FRET biosensor based on miRNA-directed intracellular self-assembly of chiral nanorod dimers (Xu et al., 2018).



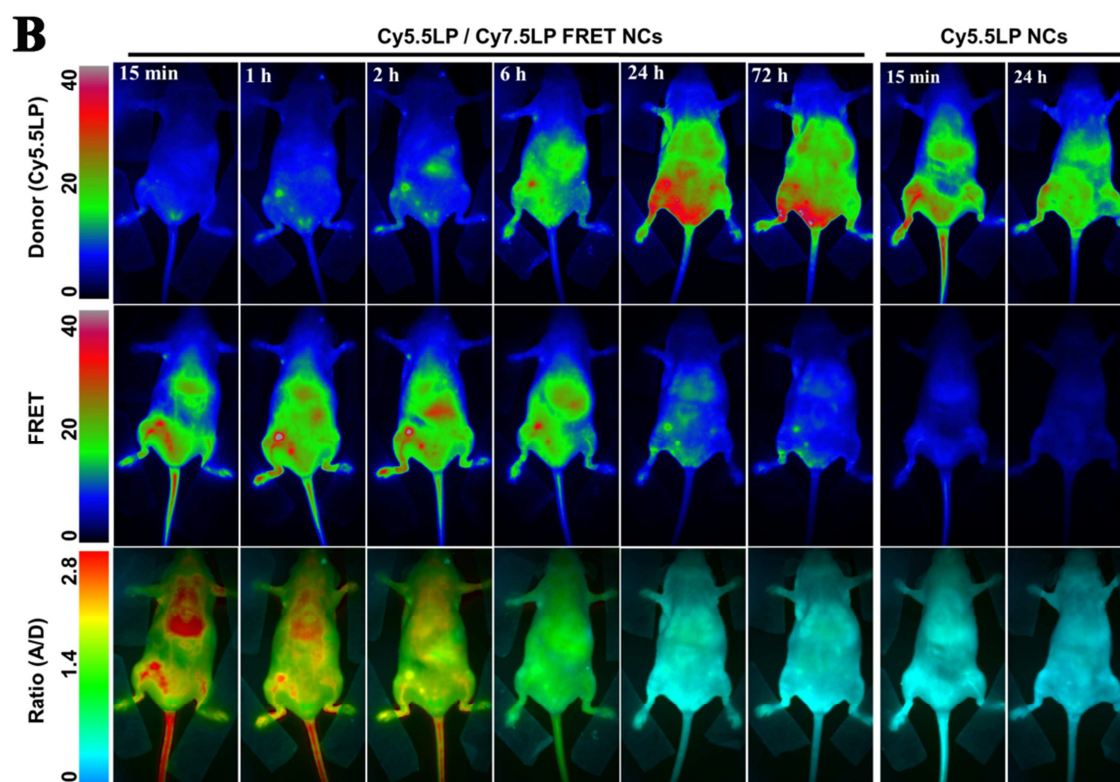


Figure 7. Integrity of lipid nanocarriers in bloodstream and tumor quantified by near-infrared ratiometric FRET imaging in living mice. Reprinted (adapted) with permission from Bouchaala et al. (2016).

3.5. Tissue-based biosensor

FRET has also been successfully used for tissue-based biosensor (Bouchaala et al., 2016; Mei et al., 2007; Campas et al., 2008; Guo and Tan, 2010; Chen et al., 2011; Guo et al., 2018). Bouchaala fabricated a fluorescent nano-emulsion droplets (NDs) of 100 nm size by encapsulating lipophilic near-infrared cyanine 5.5 and 7.5 dye into nano-emulsion droplets with the help of hydrophilic counterion tetraphenylborate for *vivo* imaging as shown in Figure 7 (Bouchaala et al., 2016). This biosensor indicated that the integrity of the FRET NCs in the blood circulation of healthy mice is preserved at 93% at 6 h of post-administration, while it drops to 66% in the liver (half-life is 8.2 h). Chen et al fabricated an acid-active tumor targeting nanoplatfrom 2,3-dimethylmaleic anhydride (DA)-trans-activating transcriptional activator (TAT)-poly(ϵ -caprolactone) (PEG-PCL, PECL) to inhibit the nonspecific interactions of TAT in the bloodstream. The FRET signal between camptothecin (CPT) and maleimide

thioether bond is monitored to visualize the drug release process (Guo et al., 2018).

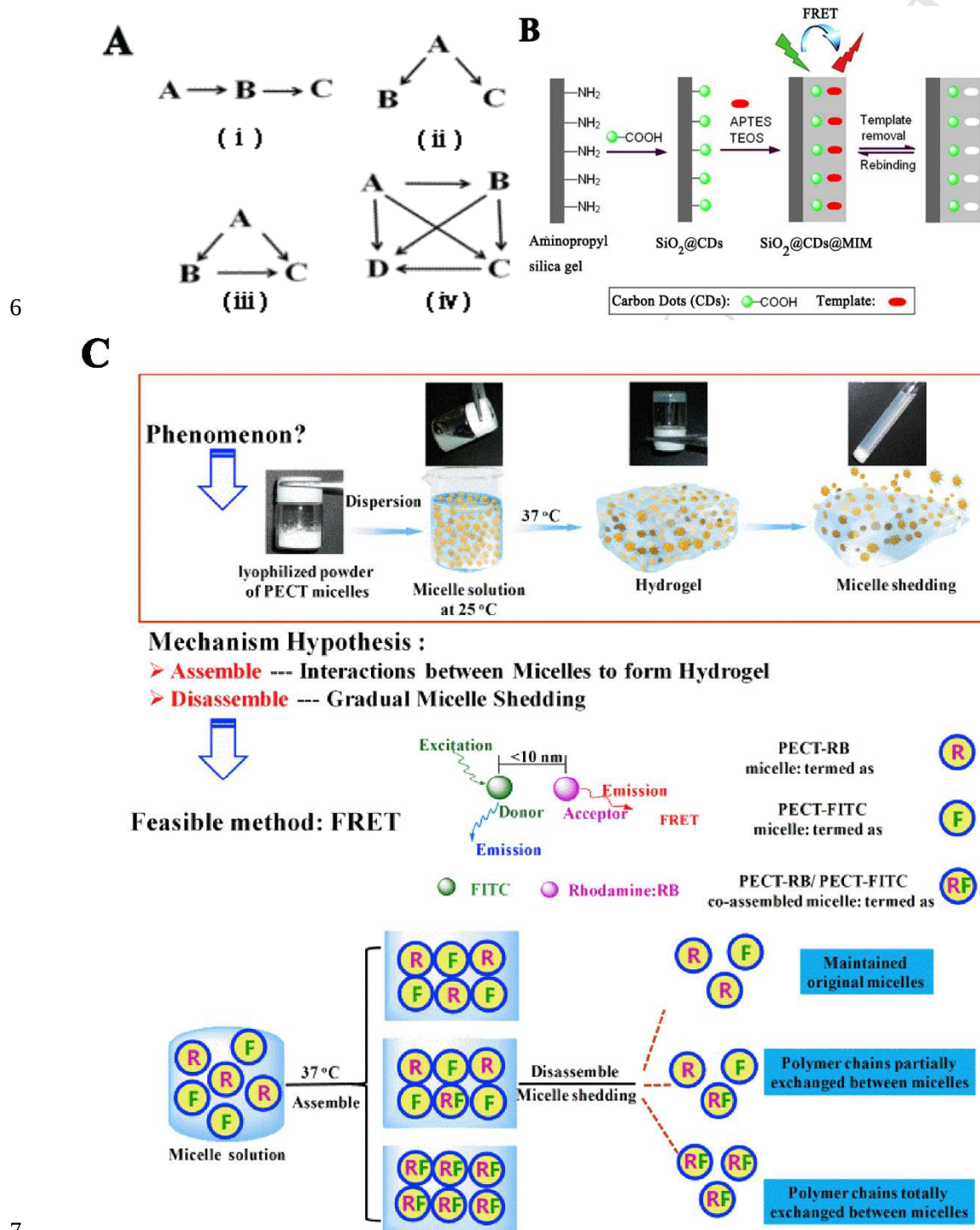
3.6. Other FRET sensors

Although the distance from the donor to the acceptor is the main effect in the FRET system, a multi-FRET biosensor with independent distance was also reported. Saha reported a multi-step FRET system for the study of molecular level by using three dyes, pyrene (Py), Acriflavine (Acf) and Rhodamine B (RhB) as shown in Figure 8A (Saha et al., 2016). As an energy transfer platform, Acf could receive energy from Py and then transfer energy to RhB. This multi-step FRET process has advantageous merits for the studying molecular level interaction beyond conventional FRET distance (1-10 nm) and multi-branched macromolecules. And the incorporation of nanoclay laponite could enhance the transfer efficiency of FRET.

Moreover, a molecularly imprinted fluorescent biosensor based on FRET was developed for the detection of doxorubicin. This system was utilized doxorubicin as the template to fabricate a molecularly imprinted polymer thin layer onto the surface of modified silica nanosphere modified by CD through sol-gel polymerization. The FRET biosensor showed high sensitivity and selectivity for doxorubicin with the detection limit of 13.8 nM (Xu et al., 2017).

FRET has also been used for thermal biosensor (Kearney et al., 2013; Conde et al., 2016; Kim et al., 2012; Zhou et al., 2015), where biosensors were assimilated into a physical transducer. Huang fabricated a FRET nano-platform for monitoring thermos-sensitive nanoscale assembly of polymeric micelles into hydrogel and release process of micelles (Huang et al., 2017). The thermo-sensitive “micellar hydrogel” was prepared based on poly(ϵ -caprolactone-co-1,4,8-trioxa [4.6] spiro-9-undecanone)-b-poly(ethyleneglycol)-b-poly(ϵ -caprolactone-o-1,4,8-trioxa[4.6] spiro-9-undecanone) (PECT) triblock copolymer in Figure 8C. In this system, FITC as a donor and rhodamine B (RB) as an acceptor were separately conjugated to PECT polymers to obtain the PECT-FITC and PECT-RB nanoparticle. The micelles co-assembled by PECT-FITC and PECT-RB polymers within the range of 10 nm will generate strong emission at 590 nm at excitation of 465 nm. This hydrogel was

1 injected into rat to tract the local delivery and degradation of micelles through FRET
 2 imaging. The thermos-sensitive biosensor facilitates the hydrogel formation and the
 3 sustained shedding of cognate micelles (Huang et al., 2017). By using the
 4 multiple-tags based FRET-imaging technology, the fate of macro biodegradable
 5 materials in vitro and in vivo can be followed at a precise nano even molecular level.



8 **Figure 8.** (A) Multi-step FRET biosensor for studying molecular level interaction

from Pyrene (Py) to Rhodamine B (RhB) via Acriflavine (Acf). Reprinted (adapted) with permission from Saha et al. (2016). (B) Molecularly imprinted FRET biosensor for selective and sensitive detection of doxorubicin. Reprinted (adapted) with permission from Xu et al. (2017). (C) FRET biosensor for the monitoring of the thermosensitive nanoscale assembly of polymeric micelles into macroscale hydrogel and sequential cognate micelles release. Reprinted (adapted) with permission from Huang et al. (2017).

4. Conclusions

FRET-based biosensors have been exploited for quantitative analysis of small molecular *in vitro*, real-time monitoring the dynamics of the compound in/on the surface of living cells and *in vivo* for biological applications. And their applications have also been extended to the medical diagnostics. Nanomaterial-based biosensors possess great attractive prospects in clinical diagnosis, food analysis, process and environmental monitoring. The successful immobilization of the fluorophore into nanomaterial could present an advantage of stability in the FRET system. FRET biosensors, as the advantageous non-invasive procedure, are valuable imaging methods for visualization of specific molecular activity during significant cellular process. The development of novel FRET system, such as multi-FRET system, and the specific receptor for the analysis would be beneficial for the fabrication of biosensors. The applications of FRET-based biosensors for cellular processes, disease diagnostics, and medical drug therapy, will undoubtedly be of great significance.

5. Future Perspectives

Extensive researches have been demonstrated that FRET-based biosensors are promising and non-invasive tools for monitoring the dynamic change of the small-molecular, proteins and living cell in laboratory environments. Despite of the various applications, FRET-based biosensors have many significant challenges in high-fluorescent resolution, improved specificity of the reaction, and the affordability of FRET reagents. For example, the strategy for enhancing the ratio of signal/noise is

major challenge. Governing the surface property of the nanoscale composites by a novel functional group is also great challenge for the presently existing techniques. Moreover, dynamic monitoring for physiological conditions in real time reversibly is also major impediments. Future work should focus on the fabrication of new FRET system such as multi-FRET biosensor. And more fluorophores in multi-step FRET system should be chosen for the detection of some larger molecular. Besides, the development of FRET has appeared many of variation, including bioluminescence and chemiluminescence resonance energy transfer, and which would be beneficial for the biological research.

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1. This paper reviews the biosensors based on FRET for the drug testing, cell imaging, molecular biology and medical diagnosis.
2. The dual-FRET systems and multi-FRET systems for biological application are also introduced
3. We present the prominent roles of nanomaterials in the development of more sensitive and specific FRET biosensors
4. We also emphasized on the challenges and outlooks of FRET biosensors.