31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Engineering NIR Dyes for Fluorescent Lifetime Contrast

Mikhail Y. Berezin[1], Hyeran Lee[1], Walter Akers[1], Kevin Guo[1], Reece J. Goiffon[1], Adah Almutairi[2], Jean M. J. Fréchet[3], Samuel Achilefu[1,4]

Abstract— The excited state of an organic molecule is a crossroads which can lead to many directions, such as non-radiative emission as heat, fluorescence, intersystem crossing and phosphorescence. Due to the unpredictable nature of the excited molecular structure, manipulation of this represents significant challenges for physicists and chemists. However, the successful management of the excited state provides a number of benefits with innumerable applications to fields like photonics and medicine. One such property of the excited state with powerful ramifications in medical diagnostics is fluorescence lifetime.

I. Introduction

The fluorescence lifetime of a molecule can be loosely defined as the average amount of time the molecule spends in its excited state. When the molecule absorbs a photon through its photochromic system, one of the electrons is promoted to an excited state and through intersystem crossing, descends to the first excited state vibrational orbital. The molecule can stay in this state for a relatively long period of time, from hundreds of picoseconds to tens of nanoseconds. The fluorescence lifetime depends not only on its structure in the excited state, where rigidity is the most important parameter, and its environment, such as polarity, viscosity, pH, temperature and oxygen concentration. Since all these parameters are biologically relevant and their abnormalities often associated with to diseases, application of fluorescent lifetime as an imaging modality in medical biology is rapidly growing. Herein, we present fluorescent lifetime imaging based on the changes around polarity of the probes and its applications for in vivo. We will begin at the synthesis of the NIR compounds and move to their lifetime properties. We report the synthesis of the NIR compounds and their lifetime properties. We also demonstrate how the lifetime can be affected by changes in polarity and used in providing information about biological processes in live animals.

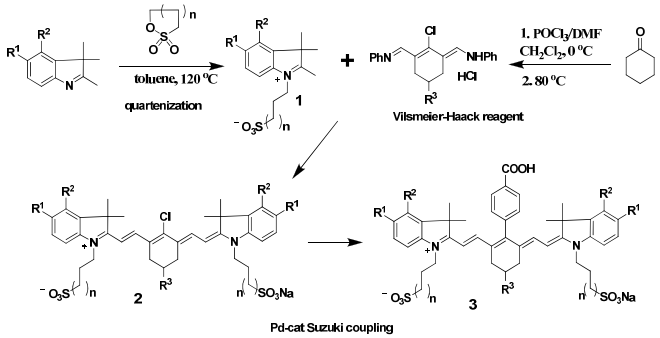
Manuscript received June 19, 2009. This work was supported in part by the National Institutes of Health (R01 CA109754, R01 EB1430, RO1 EB7276 and U01 HL080729)).

Ccorresponding author – Mikhail Y. Berezin: 314-747-0701; fax: 314-747-5191; e-mail: berezinm@mir.wustl.edu).

[1]Washington University School of Medicine, Department of Radiology, St. Louis, MO 63110, [2]UCSD Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, [3]Department of Chemistry, University of California, Berkeley, CA 94720, [4]Washington University School of Medicine, Department of Biochemistry and Molecular Biophysics, St. Louis, MO 63110

II. Synthesis of the NIR Lifetime Fluorescent Dyes

The synthesis of a NIR probes suitable for in vivo lifetime imaging requires several key intermediates, such as Vilsmeier-Haack reagent (Scheme 1) which could be prepared in relatively large quantities (>25 g) in one step.



Scheme1 Synthesis of a library of NIR dyes substituted at the meso position

The reaction of this reagent with quaternary indolenines (derivatives of compound 1, Scheme 1) in acetic anhydride in the presence of sodium acetate provides an array of chloro-substituted heptamethine dyes 2.

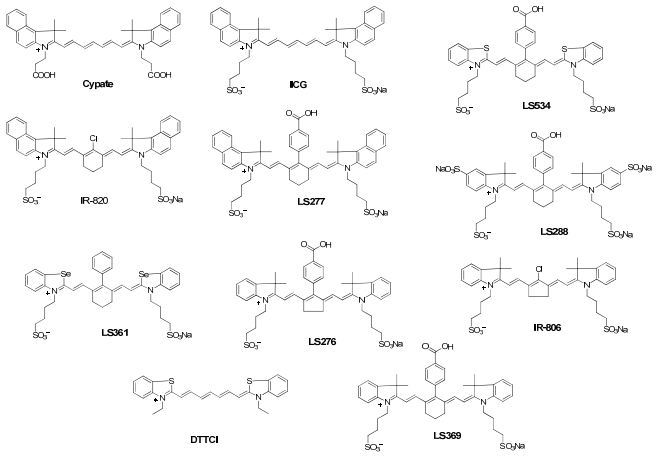


Figure 1 Structures of commercially available (IR series, ICG and DTTCI) and prepared in our laboratory (LS series and cypate) NIR dyes used in this study

Pd catalyzed Suzuki coupling of 2 further furnishes a variety of dyes with substituted meso position 3 reactive sites for conjugation to other functionalities. Examples of dyes obtained using this strategy is shown in Fig. 1. These compounds belong to heptamethine dyes which are active in

NIR range absorbing at 700-800 nm and emitting at 750-850 nm. These dyes exhibit relatively high molar extinction coefficients (> 100,000 M^{-1} cm^{-1}) and a low to moderate quantum yields (0.01-0.2 in water). The spectral and lifetime properties of the NIR dyes used in this study in different solvents are summarized in Table 1.

Table 1 Spectroscopic properties and lifetimes of NIR dyes in solvents with different polarities

		Cypate	ICG	IR-820	LS277	LS369
Abs (nm, methanol)		792	792	820	800	765
Em (nm, methanol)		817	817	836	811	780
t (ns)	water	0.20	0.17	0.13	0.20	0.44
	methanol	0.46	0.51	0.25	0.61	0.98
	ethanol	0.57	0.62	0.38	0.69	1.25
	acetone	0.80	0.87	0.43	0.84	1.13
	DMSO	0.87	0.97	0.50	0.98	1.48
	DCM	0.94	0.91	0.59	1.34	1.24
	chloroform	1.01	1.14	0.73	1.62	1.30

		LS534	LS361	LS288	LS276
Abs (nm, methanol)		800	800	770	797
Em (nm, methanol)		813	813	790	816
t (ns)	water	0.33	0.36	0.44	0.36
	methanol	0.72	0.73	0.81	0.83
	ethanol	0.93	0.93	1.00	0.87
	acetone	1.08	1.14	1.02	1.13
	DMSO	1.26	1.28	1.33	1.11
	DCM	1.19	1.29	n/a	1.17
	chloroform	1.31	1.29	n/a	1.25

III. Fluorescence Lifetime Polarity Studies

Please check with your editor on whether to submit your manuscript by hard copy or electronically for review. If hard copy, submit photocopies such that only one column appears per page. This will give your referees plenty of room to write comments. Send the number of copies specified by your editor (typically four). If submitted electronically, find out if your editor prefers submissions on disk or as e-mail attachments.

The fluorescence lifetime dependence of the dyes on various solvents was evaluated to provide insights into the excited state decay pathway. While it is generally accepted that photoisomerization decay pathway is solvent dependent and that internal conversion is a solvent independent process, it is not clear which of the solvent properties, including viscosity, polarity, or hydrogen bonding has a dominant effect on the decay pathway. To further complicate the problem, several studies of the lifetime dependence of cyanine dyes on solvent viscosity have reported contradictory results [1,2] Our previous studies showed that cyanine dyes are mostly insensitive to solvent viscosity but highly sensitive to the solvent polarity [3]. Accordingly, we determined the fluorescent lifetimes of these dyes in various solvents with different polarities. In general, the lifetime increased from the polar solvents to the non-polar solvents in the order water < MeOH < EtOH < Acetone < DMSO < DCM < CHCl3. To understand the decay pathway for these dyes, we determined the theoretical maximum fluorescent lifetime of ICG based on the known theoretical relationship between absorption intensity and fluorescence lifetime (Strickler-Berg equation [4])

The Strickler-Berg equation is based on several assumptions, one of which is the absolute rigidity of the molecule in both the ground and excited state. Thus, this approach provides an estimate of possible maximum lifetime value of a molecule restricted from any rotations. The calculated theoretical lifetime value was 3.5 ns compared to the experimental value of 0.97 ns indicating that over 70% of the excited state energy is lost through non-radiative pathways due to the lack of structural rigidity for these dyes in the excited state. Thus, it is evident that the major decay pathway for heptamethine cyanine dyes is via non-radiative photoisomerization process or torsional rotation. That suggests that rigidifying the structural framework of heptamethine dyes is an approach to design cyanine-type molecule with long lifetimes. It also suggests that the difference in polarity around the fluorophore affects the rigidity of the molecule in the excited state. The latter provides quantitative foundation for using lifetime in imaging.

IV. Correlation Between In Vitro and In Vivo Imaging

For better understanding fluorescent lifetime maps in vivo we needed to establish a correlation between the lifetime probes in vivo and in vitro. Since biological media is inherently heterogeneous the challenge was to identify a model solvent system. A model system would allow development of a high throughput in vitro model to predict the behavior and to sense normal and abnormal physiological differences by fluorescence lifetime changes in vivo. To establish the correlation between the in vitro and in vivo results we evaluated the lifetimes of eight near-infrared fluorescent molecular probes in vitro in various solvent media. We then measured the lifetimes of these dyes after intravenous injection in mice using time-domain diffuse optical imaging, creating whole-body lifetime maps. The lifetimes of NIR probes in various solvents with different polarity indices were compared to the in vivo data to develop a scale of lifetime sensitivity. Two exponential fitting of the fluorescence decays of dyes dissolved in aqueous albumin solutions more accurately predicted the range of lifetimes observed in vivo. We found that the mean lifetime in aqueous albumin solution was close to the maximum value of the lifetime in vivo, while the minimum value was close to polar solutions such as water.

Table 2 Fluorescent lifetimes (ns) of NIR molecular probes measured in various solvents and after intravenous administration in living mice. Data were fitted by a single exponential decay model

	chloroform	DCM	DMSO	acetone	ethanol
LS288	N/A[c]	N/A	1.33	1.02	1.00
LS276	1.25	1.17	1.11	1.13	0.87
DTTCI	1.82	1.72	1.49	1.54	1.21
IR-806	1.28	1.15	1.01	1.02	0.82
LS277	1.62	1.34	0.98	0.84	0.69
ICG	1.14	0.91	0.97	0.87	0.62
Cypate	1.01	0.94	0.87	0.8	0.57
IR-820	0.73	0.59	0.50	0.43	0.38

	methanol	water	albumin	in vivo
LS288	0.81	0.44	1.00	1.12
LS276	0.83	0.36	0.88	1.12
DTTCI	1.07	0.49	1.14	0.89
IR-806	0.66	0.27	0.61	0.77
LS277	0.61	0.2	0.78	0.74
ICG	0.51	0.17	0.79	0.69
Cypate	0.46	0.2	0.62	0.63
IR-820	0.25	0.13	0.49	0.53

V. Engineered Lifetime Probes for Biomedical Applications

A. Fluorescent lifetime of NIR dyes as potential reporter of nanoparticles degradation in vivo

Synthetic polymers and dendrimers have been widely used by the medical community to overcome biological barriers and enhance in vivo biomedical applications. Despite the widespread use of biomaterials it has been generally difficult to monitor their fate in vivo. In this example fluorescence lifetime imaging was used to assess the stability of the nanoparticles in live animals. For that a multilayered nanoprobe, consisting of a near infrared core, nano-encapsulated in a biodegradable dendrimer, and surrounded by a shell of polyethylene oxide (Fig. 2) was administered to a mouse. Covalent encapsulation of the near infrared fluorophores in the dendritic scaffold conferred enhanced stability to the nanoprobe with added resistance to enzymatic oxidation and prolonged blood residence time. Insight into the time course of biodegradation of this nanoprobe was gained using non-invasive whole body in vivo fluorescence lifetime imaging.

Prior to administering the nanoparticles into mice, the optical properties of nanoparticles were studied in vitro in water (Table 3). Incorporation of the fluorophore in a polymeric matrix did not change steady-state absorption and emission properties, and slightly altered the fluorescent lifetime from 0.2 to 0.3 ns. Importantly, fluorescence lifetime of the nanoparticles became independent from the environment as opposed to naked fluorophores (cypate or ICG) and therefore could be potentially used as an imaging modality to monitor degradation of the nanoparticles.

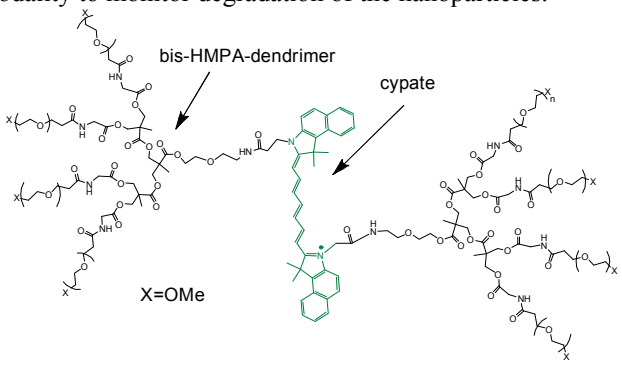


Figure 2 Structure of a biodegradable dendrimer with NIR core

Table 3 Fluorescence properties of nanoparticles and ICG *in vitro* (water) and *in vivo* (liver)

System	λ_{ex} max (nm)	λ_{em} max (nm)	τ (ns)
Cypate-core stealth nanoparticles in water	780	810	0.30
ICG in water	779	809	0.17
Cypate in water	783	810	0.20
Cypate-core stealth nanoparticles in vivo (10 mi after injection)			0.35
ICG in vivo (10 min after injection)			0.69

Whole-body fluorescence lifetime maps, shown in Fig. 3, were created from time-domain whole-body fluorescence imaging at indicated time points after injection of the nanoprobes. In vivo fluorescence lifetime changes over a seven day period allowed us to non-invasively monitor the degradation kinetics of the dendritic nanoprobe in real time.

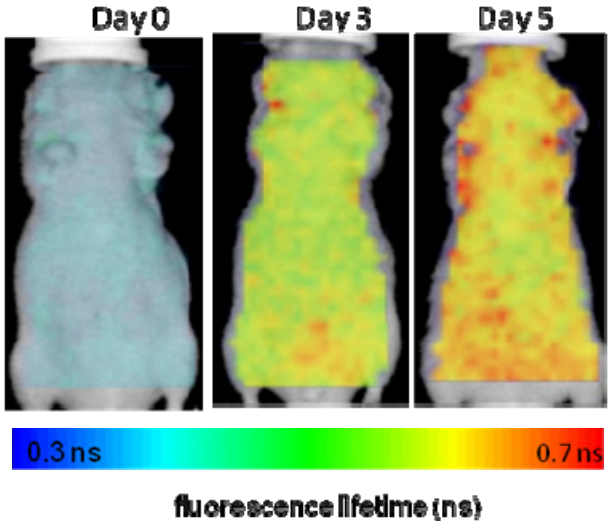


Figure 3 Fluorescent lifetime maps acquired by time-domain optical imaging after intravenous administration of biodegradable dendrimer with NIR core at indicated times after injection

No changes in the fluorescence lifetime images over time were observed when using cypate throughout the duration of imaging.[5] The increase of fluorescence lifetime from 0.35 ns immediately after injection to 0.7 ns on day 5 clearly indicated the decompositions of the nanoparticles. We suspect the hydrolysis of this aliphatic dendrimer leads to exposure of the NIR fluorophore which is then rapidly opsonized by albumin hydrophobic binding sites. Such change in polarity from high (hydrophilic PEGylated environment) to low (albumin) is reflected by the changes in fluorescence lifetime.

B. Fluorescent lifetime of NIR dyes as potential reporter of diseases

From in vitro data obtained using a number of NIR molecular probes, we have demonstrated that the different lifetimes in vivo depend on the nature of the molecular probe. In general, dyes with longer fluorescent lifetimes in any given solvent generally exhibited longer lifetime values in vivo. Data analysis of lifetimes in various media identified albumin solution as a consensus model for predicting the in vivo lifetimes relative to the other media. This finding is supported by the fact that cyanine dyes are rapidly opsonized by plasma proteins such as serum albumin soon after injection.[6] Thus, a majority of the lifetime values observed in vivo reflected the lifetime of NIR dyes bound to serum albumin.

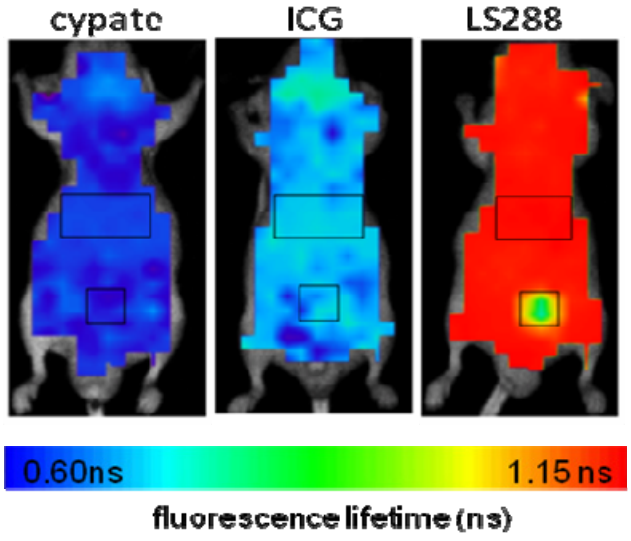


Figure 4 NIR dyes with corresponding fluorescence lifetime maps acquired within 30 minutes after intravenous administration. Liver and bladder ROIs for fluorescent lifetime analyses are indicated by boxes.

The preferred route of elimination after intravenous administration was observed within a few minutes after injection. ICG and cypate have high binding affinity to albumin and stay in blood and eliminated via the hepatobiliary system. Fluorescence lifetime signal for these tissues is small resulting in relatively uniform fluorescence lifetime map. In contrast, the fluorescence from LS288 can be seen from both the liver and the bladder area (Fig. 4), demonstrating intermediate attributes between hepatic and renal elimination. LS288 are quickly filtered by the renal system into the urine. Renal filtration of these dyes is demonstrated in Fig. 4 by the distinct drop in fluorescent lifetime. Indeed, the lifetime of LS288 in urine is markedly different from albumin and correlates well with the lifetime in corresponding organs in vivo (Table 4).

Table 4 Measured lifetime (ns) of LS288 in vitro and in vivo comparing the protein-bound fractions in albumin solution and in the liver ROI to that of relatively water-rich urine

in vitro		in vivo	
Albumin	1.03	Liver	1.06
Urine	0.51	Bladder	0.63

Kidneys normally filter the blood of excess salts and metabolic products such as urea while retaining plasma proteins. In diseases such as multiple myeloma and diabetes mellitus, the renal function is compromised and protein escapes into the urine. The lifetime of LS288 in urine could be to image excess serum protein loss in urine (proteinuria) which was demonstrated by direct injection of albumin into bladder which leads to the immediate increase of the LS288 lifetime. These results indicate that lifetime can be useful for the dynamic imaging of protein-losing nephropathy due renal diseases and suggest the potential use of the fluorescent lifetime imaging to distinguish tumors from fluid-filled cysts in the body.

REFERENCES

[1] Soper, S. A. & Mattingly, Q. L. Steady-State and Picosecond Laser Fluorescence Studies of Nonradiative Pathways in Tricarbocyanine Dyes - Implications to the Design of near-Ir Fluorochromes with High Fluorescence Efficiencies. J. Am. Chem. Soc. 116, 3744-3752 (1994).

[2] Kasatani, K. & Sato, H. Viscosity-dependent decay dynamics of the S-2 state of cyanine dyes with 3, 5, and 7 methine units by picosecond fluorescence lifetime measurements. Bull. Chem. Soc. Jpn. 69, 3455-3460 (1996).

[3] Berezin, M. Y., Lee, H., Akers, W., Nikiforovich, G. & Achilefu, S. Fluorescent lifetime of near infrared dyes for structural analysis of serum albumin. Proc. SPIE 6867, 68670J-68670J-68611 (2008).

[4] Strickler, S. J. & Berg, R. A. Relation between absorption intensity and fluorescence lifetime of molecules. J. Chem. Phys. 37, 814-822 (1962).

[5] Bloch, S. et al. Whole-body fluorescence lifetime imaging of a tumor-targeted near-infrared molecular probe in mice. J Biomed Opt 10, 0540031-0540038 (2005).

[6] Cherrick, G. R., Stein, S. W., Leevy, C. M. & Davidson, C. S. Indocyanine green: observations on its physical properties, plasma decay, and hepatic extraction. J. Clin. Invest. 39, 592-600 (1960).