Development of fluorescent tracers for the real-time monitoring of renal function

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ABSTRACT

Accurate measurement of glomerular filtration rate (GFR) at the bedside is highly desirable in order to assess renal function in real-time, which is currently an unmet clinical need. In our pursuit to develop exogenous fluorescent tracers as GFR markers, various hydrophilic derivatives of 3,6-diaminopyrazine-2,5-dicarboxylic acid with varying molecular weights and absorption/emission characteristics were synthesized. These include polyhydroxyalkyl based small molecules and poly(ethylene glycol) (PEG) substituted moderate molecular weight compounds, which were further sub-grouped into analogs having blue excitation with green emission, and relatively longer wavelength analogs having green excitation with orange emission. Lead compounds were identified in each of the four classes on the basis of structure–activity relationship studies, which included in vitro plasma protein binding, in vivo urine recovery of administered dose, and in vivo optical monitoring. The in vivo optical monitoring experiments with lead candidates have been correlated with plasma pharmacokinetic (PK) data for measurement of clearance and hence GFR. Renal clearance of these compounds, occurring exclusively via glomerular filtration, was established by probenecid blocking experiments. The renal clearance property of all these advanced candidates was superior to that of the iothalamate, which is currently an accepted standard for the measurement of GFR.

Keywords: Glomerular filtration rate (GFR), renal function, fluorescence, pyrazine, PEG, optical monitoring, renal clearance, probenecid

1. INTRODUCTION

The measurement of glomerular filtration rate (GFR) is widely accepted by the medical community as the best indicator of kidney function, and is a key component in the diagnosis and management of renal impairment.[1] A simple and accurate method for determining GFR in real-time in a non-invasive fashion is highly desirable, and is currently an unmet clinical need. In clinical practice, GFR is not measured directly, but is estimated using equations based on a single serum creatinine measurement, or in conjunction with measurement of creatinine clearance from urine collected over a 24 hour period. Though measurement of serum creatinine as an indicator of renal function is a practical method under current circumstances, it lacks sensitivity, and may be misleading because it is affected by age, gender, muscle mass, dietary intake, and many other anthropometric variables.[2–4] The most accurate methods for measurement of GFR demand complex techniques involving the use of exogenous tracer agents such as inulin,[5] iothalamate,[6] and ^{99m}Tc-DTPA.[7] To overcome some of the limitations such as laborious ex-vivo handling of blood and urine samples and use and disposal of radioactivity, considerable effort has been directed at developing fluorescent GFR agents that can be utilized for real-time point-of-care monitoring of renal function.[8–11]

During the course of our investigations in developing exogenous fluorescent GFR markers, we have identified 3,6-diaminopyrazine-2,5-dicarboxylic acid (**1**) and its analogs as photostable small molecules that possess highly desirable photophysical properties.[12,13] Pyrazine derivatives that contain electron donating groups at 3,6-positions and electron

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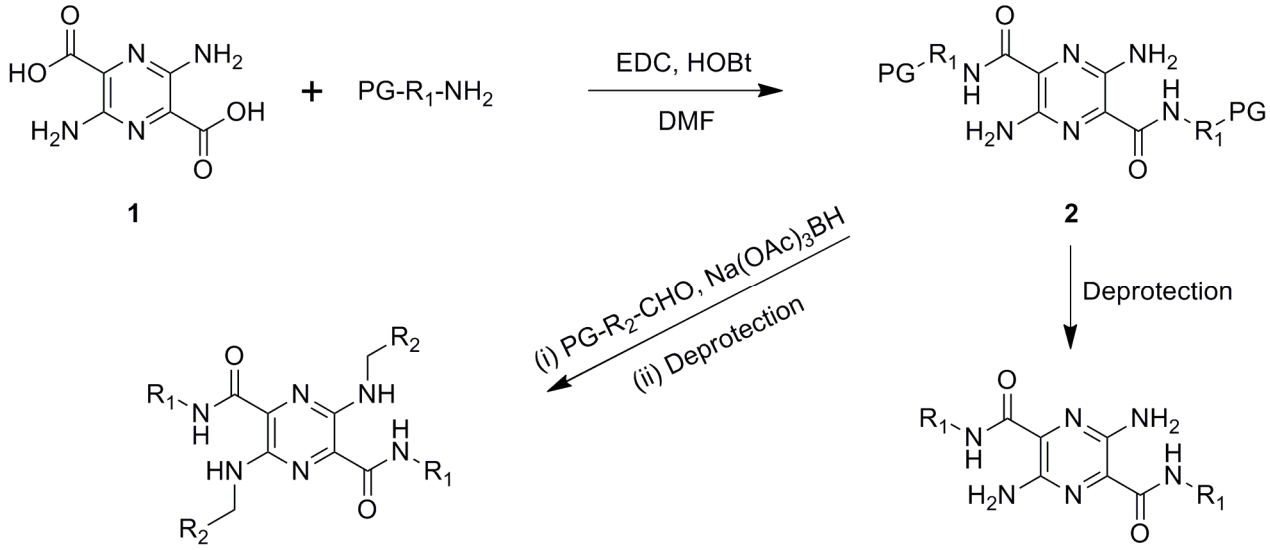
withdrawing groups at 2,5-positions strongly absorb and emit in the blue to orange regions of the electromagnetic spectrum with large Stokes' shift. The extreme insolubility of most of these known dyes in aqueous media renders them unsuitable for development as GFR tracers. However, the diacid **1** is an excellent scaffold for further modification as it can be functionalized at both amino and carboxyl terminals. Consequently, several hydrophilic amide derivatives of **1** containing amino acid, poly (ethylene glycol) (PEG), and polyhydroxyalkyl groups were designed and synthesized. In this paper, we present their structure–activity relationship (SAR) studies and the selection of lead candidates for further development for noninvasive monitoring of renal function.[14,15]

2. MATERIALS AND METHODS

2.1 Polyhydroxyalkylated pyrazines

Preparation of various polyhydroxyalkylated pyrazines is outlined in Scheme 1. The diacid **1** was reacted with protected amines PG-R_1-NH_2 (PG = protecting group) with EDC–HOBt coupling to give amides **2**, which under standard deprotecting conditions afforded the corresponding derivatives **3a–d**. The protecting groups used were *tert*-butyl, acetonide, benzyl ester, and dibenzyl ester respectively in the synthesis of compounds **3a–d**. Dilute HCl was utilized to cleave *tert*-butyl and acetonide groups and hydrogenolysis was employed in the removal of benzyl esters. The N-alkylated pyrazine derivatives **4a–d** were prepared by initial reductive amination[16] of the appropriate amide **2** with protected aldehydes in 1,2-dichloroethane (DCE) followed by relevant deprotection method. For compound **4d**, acetonides were cleaved prior to the removal of benzyl esters. It should be mentioned here that compound **4a** was prepared from the known 3,6-diamino-2,5-dicarboxamide **2** (PG-R_1 = H).[17]



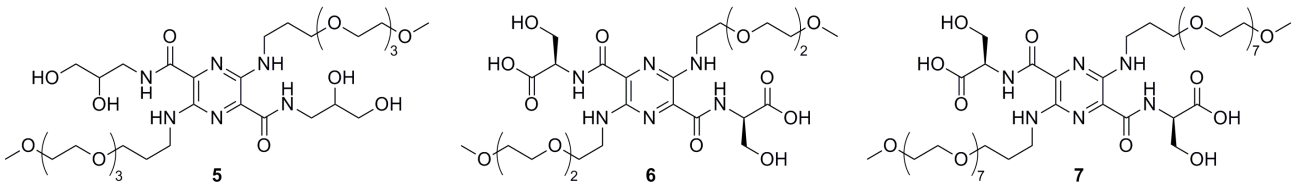
4a: R_1 = H, R_2 = *CH(OH)CH_2OH (*S,S*-Isomer)
4b: R_1 = CH_2CH(OH)CH_2OH, R_2 = *CH(OH)CH_2OH (*S,S*-Isomer)
4c: R_1 = *CH(CH_2OH)CO_2H, R_2 = CH_2CH_3 (*R,R*-Isomer)
4d: R_1 = *CH(CH_2OH)CO_2H, R_2 = *CH(OH)CH_2OH (*R,R,S,S*-Isomer)

3a: R_1 = CH_2CH_2OH
3b: R_1 = CH_2CH(OH)CH_2OH
3c: R_1 = *CH_2(CH_2OH)CO_2H (*R,R*-Isomer)
3d: R_1 = *CH(CO_2H)CH_2CO_2H (*R,R*-Isomer)

Scheme 1. Synthesis of polyhydroxyalkyl based small molecule pyrazine derivatives.

2.2 PEGylated hydroxyalkylpyrazines

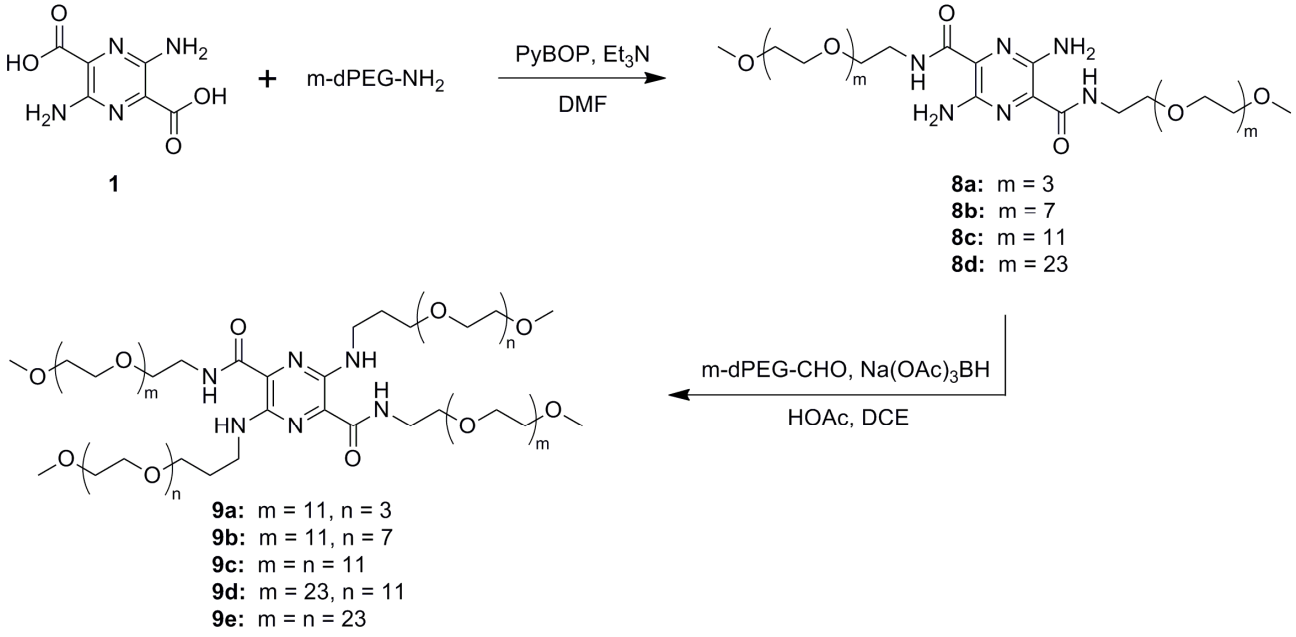
Compounds **5–7** that are decorated with both polyhydroxyalkyl/dianionic- and small PEG-groups were prepared according to the method described in Scheme 1 for the synthesis of **4**. The carboxylic groups of the diacid **1** were initially coupled to appropriate protected amine followed by reductive alkylation with the desired discrete methoxypoly(ethylene glycol) aldehyde. Subsequent removal of the protecting groups afforded the compounds **5–7** (Scheme 2).



Scheme 2. Synthesis of polyhydroxyalkylated /PEGylated pyrazine derivatives.

2.3 PEGylated pyrazines

Preparation of pyrazine-PEG conjugates is depicted in Scheme 3. The coupling of diacid **1** with discrete methoxypoly(ethylene glycol) amines was achieved by PyBOP, which was found to be superior reagent when compared to EDC–HOBt leading to the formation of **8a–d** in good yields. The carboxamides **8c** and **8d** were then reductively alkylated with various discrete m-PEG-aldehydes to give the corresponding N-alkylated conjugates **9a–e**. All the PEG-conjugates were purified by reverse phase preparative HPLC using 0.1% TFA modifier in a water–acetonitrile gradient.



Scheme 3. Synthesis of PEGylated pyrazine derivatives.

2.4 In vitro characterization and in vivo studies

In general, a 2 mM stock solution of each compound was prepared in PBS buffer and diluted further as needed. The absorption spectra were measured at a concentration of 100 μM and fluorescence properties were determined on a 10 μM solution. The protein binding was obtained on a 20 μM compound solution in rat plasma incubated at 37 °C for 1 h. After separating free compound from bound via cellulose membrane (30,000 MWCO), the concentration of protein-free was determined by HPLC analysis using a set of external calibration standards and fluorescence detection. For urine

recovery studies, the test compound (1 mL) was administered by tail vein injection into conscious, restrained Sprague-Dawley rats, with subsequent collection of urine at the time points of 2, 4 and 6 hours post injection. Alternatively, rats were anesthetized with 100 mg/kg Inactin (I.P.) and a trachea tube was inserted to maintain respiration prior to the injection of test compound into the lateral tail vein, and the abdomen was opened after 6 hours to remove urine from the bladder. Quantitation of each compound in urine was performed via HPLC analysis and the percent recovery of compound at each time point was calculated based on the balance of mass. Non-invasive optical monitoring studies were performed on male Sprague-Dawley rats that were anesthetized by Inactin (I.P.) or 2% isoflurane. The test compound (1 mL) was injected into the tail-vein of the rat and the fluorescence signal was monitored at the ear by placing it near the common end of the bifurcated fiber optic bundle that was attached to a laser source and a detector system.[18] Invasive pharmacokinetic studies were carried out on male Sprague-Dawley rats anesthetized by Inactin (I.P.) and surgically instrumented with a trachea tube to facilitate breathing and femoral artery and vein catheters for blood sampling and drug administration respectively. After administration of test compound (1 mL), approximately 200 μL blood was sampled and placed into a heparinized tube at 0, 1, 6, 12, 18, 30, 45, 60, 90, 120 minutes. The concentration of compound in each centrifuged plasma sample was determined via HPLC analysis and the resulting pharmacokinetic parameters of the compound were analyzed using WinNonLin pharmacokinetic modeling software. A similar procedure was utilized in probenecid inhibition studies, the only difference being that the rats received 70 mg/kg dose of probenecid 10 minutes prior to the test compound.

3. RESULTS AND DISCUSSION

3.1 Photophysical properties, protein binding and urine recovery studies

The absorbance (λ_{abs}) and emission (λ_{em}) maxima and plasma protein binding (PPB) were measured for all the compounds. Also, urine recovery profiles were determined prior to conducting non-invasive optical clearance experiments for selected compounds (Table 1). The pyrazine carboxamides **3a–d** and **8a–d** exhibited absorption maxima in the range of 432–438 nm (blue) and emission maxima in the range of 557–559 nm (green) respectively with large Stokes' shifts (>100 nm). The N-alkylated compounds **4a–d**, **5–7**, and **9a–e** showed bathochromic shifts (~40–60 nm) as expected and displayed absorption maxima in the range of 484–499 nm (green) and emission maxima in the range of 594–605 nm (orange) respectively. These red-shifted analogs were synthesized with view to enhancing the in vivo optical detection method due to relatively increased tissue penetration at these longer wavelengths. Most of these compounds bind minimally to plasma proteins with PPB <<10%, with sole exception being relatively lipophilic dipropyl analog **4c** with 74% PPB. In comparison, the extent of PPB is 10% for currently accepted GFR standard iothalamate.[19] On the basis of urine recovery profiles of small molecular weight pyrazine derivatives, the neutral polyhydroxy derivative **4b** and the dianionic compounds **3c** and **4d** showed better renal clearance than the standard iothalamate (85–90% vs. 80% for iothalamate). Among the PEGylated pyrazines with diamino groups, compound **8d** with two 24-mer m-PEGs showed optimal clearance properties (96%). All the N-alkylated analogs in the tetra-PEG series **9a–e**, showed better clearance profiles than iothalamate and compound **9c** with four 12-mer m-PEGs showed highest (97%) urine recovery. The urine recovery of hybrid analogs **6** and **7** containing both dianionic- and PEG-groups was found to be only 14% and 61% respectively though they exhibited no protein binding.

3.2 Optical monitoring of renal elimination and invasive PK studies

The non-invasive real-time monitoring of plasma clearance of most of the compounds that exhibited better urinary recovery when compared to iothalamate was accomplished by following the fluorescence intensity at the rat ear.[18] The typical time course for the clearance indicate a biphasic elimination profile with free distribution from blood into the tissue, and the profile conforms to at least a two compartment model (Figure 1). The small molecules **3c** and **4b** clear from the plasma with a terminal or β phase half-life ($t_{1/2}\beta$) of about 23 minutes and the PEGylated analogs **8d** and **9c–e** clear with relatively lower half-lives of 15–20 min. The relative faster clearance of PEGylated analogs could be resulting from relatively lower distribution into the tissue, i.e., lower volume of distribution due to their increased molecular size. The lower molecular weight PEG chains (<6000 Da) are known to be filtered by glomerulus and not absorbed by renal tubules,[20] and the largest of the compounds described here **9e** contains four 24-mer m-PEGs with a molecular weight of 4509 Da, which is within the desired range.

Table 1. Photophysical properties and urine recovery data of pyrazine derivatives.

Compound	Photophysical properties		Amount recovered in urine at 6 h[a] (%)
	λ_{abs} (nm)	λ_{em} (nm)	
3a	436	558	ND[b]
3b	432	558	61 ± 3 (3)[c]
3c	435	557	90 ± 1 (3)
3d	436	558	61 ± 16 (3)
4a	486	600	25 ± 4 (3)
4b	484	594	88 ± 2 (3)
4c	490	599	ND
4d	488	597	85 ± 1 (3)
5	488	607	29 ± 2 (3)
6	490	596	14 ± 1 (3)
7	495	605	61 ± 4 (3)
8a	436	557	41 ± 1 (6)
8b	438	557	45 ± 3 (3)
8c	437	558	71 ± 9 (3)
8d	439	559	96 ± 1 (6)
9a	491	603	86 ± 1 (3)
9b	498	605	87 ± 1 (3)
9c	499	604	97 ± 1 (3)
9d	499	602	89 ± 2 (3)
9e	495	603	86 ± 7 (3)
Iothalamate	NA[d]	NA	80 ± 2 (6)

[a] Given as mean ± SEM. [b] Not determined. [c] Numbers in parentheses indicate number of test animals. [d] Not applicable.

On the basis of the urine recovery and optical clearance profiles, four compounds differing in molecular size and photophysical properties were selected for invasive PK studies. These include small molecular weight compounds **3c** and **4d** and relatively larger molecular weight PEGylated analogs **8d** and **9c** respectively. Compounds **3c** and **8d** absorb in the blue region and emit in the green region, compounds **4d** and **9c** absorb in the green region and emit in the orange region of the electromagnetic spectrum respectively. In a series of separate PK experiments, there was no significant difference observed in the clearance rates of the tracers in the presence of probenecid. This indicates the route of elimination for these compounds is exclusively via glomerular filtration, since probenecid is known to block the tubular secretion pathway.[21] It should be mentioned here that though compound **4b** and **4d** exhibited similar urine clearance profiles, **4d** is preferred since it shares the same common intermediate as **3c** during the synthesis. Similarly, compound **9c** was chosen over **9d** and **9e** due to relative ease of preparation of 12-mer discrete m-PEG starting materials over the corresponding 24-mer analogs.

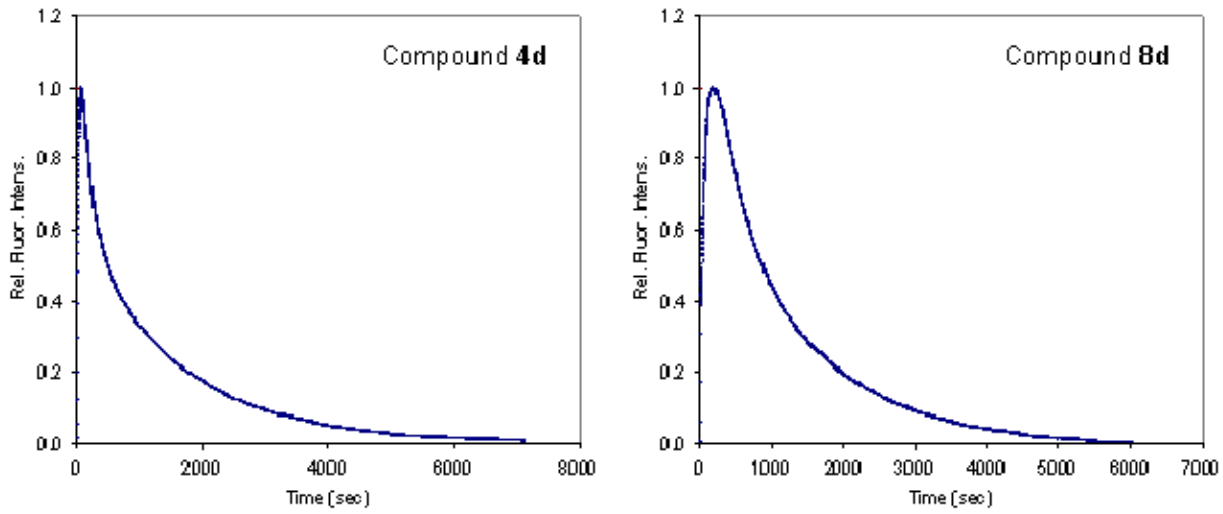


Figure 1. Non-invasive optical clearance profiles of renal agents **4d** and **8d**.

4. CONCLUSIONS

The feasibility of continuous, real-time monitoring of renal function to provide an accurate measure of GFR by optical methods has been demonstrated with pyrazine based fluorescent tracers. By careful tuning of the pyrazine scaffold for renal clearance, variety of compounds like **3c**, **4d**, **8d**, and **9c** were identified that were superior to the clinical standard iothalamate in rat models. The selection of the clinical candidate will be based on further studies that include toxicology, tissue optics properties, instrumental design, and algorithms to transform the optical clearance data to GFR.

REFERENCES

[1] Stevens, L. A. and Levey, A. S., "Measured GFR as a confirmatory test for estimated GFR," J. Am. Soc. Nephrol. 20, 2305–2313 (2009).
[2] Agarwal, R., "Estimating GFR from serum creatinine concentration: pitfalls of GFR-estimating equations," Am. J. Kidney Dis. 45, 610–613 (2005).
[3] Verhave, J. C., Fesler, P., Ribstein, J., du Cailar, G. and Mimran, A., "Estimation of renal function in subjects with normal serum creatinine levels: influence of age and body mass index," Am. J. Kidney Dis. 46, 233–241 (2005).
[4] Diskin, C. J., "Creatinine and GFR: an imperfect marriage of convenience," Nephrol. Dial. Transplant. 21, 3338–3339 (2006).
[5] Sturgeon, C., Sam, A. D. and Law, W. R., "Rapid determination of glomerular filtration rate by ingle-bolus inulin: a comparison of estimation analyses," J. Appl. Physiol. 84, 2154–2162 (1984).
[6] Wilson, D. M., Bergert, J. M., Larson, T. H. and Leidtke, R. R., "GFR determined by nonradiolabeled iothalamate using capillary electrophoresis," Am. J. Kidney Dis. 39, 646–652 (1997).
[7] Lewis, N., Kerr, R. and Van Buren, C., "Comparative evaluation of urographic contrast media, inulin, and ^{99m}Tc-DTPA clearance methods for determination of glomerular filtration rate in clinical transplantation," Transplantation 48, 790–796 (1989).
[8] Rabito, C .A., Chen, Y., Schomacker, K. T. and Modell, M. D., "Optical, real-time monitoring of the glomerular filtration rate," Appl. Opt. 44, 5956–5965 (2005).
[9] Yu, W., Sandoval, R. M. and Molitoris, B. A., "Rapid determination of renal filtration using an optical ratiometric imaging approach," Am. J. Physiol. Renal Physiol. 292, F1873–F1880 (2007).

[10] Chinen, L., Galen, K. P., Kuan, K. T., Dyszlewski, M. E., Ozaki, H., Sawai, H., Pandurangi, R. S., Jacobs, F. G., Dorshow, R. B. and Rajagopalan, R., "Fluorescence-enhanced europium complexes for the assessment of renal function," J. Med. Chem. 51, 957–962 (2008).
[11] Schock-Kusch, D., Sadick, M., Henninger, N., Kraenzlin, B., Claus, G., Kloetzer, H. -M., Weiss, C., Pill, J. and Gretz, N., "Transcutaneous measurement of glomerular filtration rate using FITC-sinistrin in rats," Nephrol. Dial. Transplant. 24, 2997–3001 (2009).
[12] Kim, J. H., Shin, S. R., Matsuoka, M. and Fukunishi, K., "Self-assembling of aminopyrazine fluorescent dyes and their solid state spectra," Dyes and Pigments 39, 341–357 (1998).
[13] Kim, J. H., Shin, S. R., Matsuoka, M. and Fukunishi, K., "Self-assembling of aminopyrazine fluorescent dyes and their solid state spectra, Part 2," Dyes and Pigments 41, 183–191 (1999).
[14] Presented in part: Dorshow, R. B., Asmelash, B., Chinen, L. K., Debreczeny, M. P., Fitch, R. M., Freskos, J. N., Galen, K. P., Gaston, K. R., Marzan, T. A., Poreddy, A. R., Rajagopalan, R., Shieh, J. -J. and Neumann, W. L., "New optical probes for continuous monitoring of renal function," Proc. SPIE 6867, 68670C-1–68670C-11 (2008).
[15] Presented in part: Poreddy, A. R., Asmelash, B., Galen, K. P., Fitch, R. M., Shieh, J. -J, Wilcox, J. M., Schoenstein, T. M., Wojdyla, J. K., Gaston, K. R., Freskos, J. N., Neumann, W. L., Rajagopalan, R., Ahn, H. -Y, Kostelc, J. G., Debreczeny, M. P., Belfield, K. D. and Dorshow, R. B., "N-Alkylated aminopyrazines as hydrophilic optical agents," Proc. SPIE 7190, 71900P-1–71900P-10 (2009).
[16] Poreddy, A. R., Asmelash, B., Neumann, W. L. and Dorshow, R. B., "A highly efficient method for the N-alkylation of aminopyrazines: Synthesis of hydrophilic red fluorescent dyes," Synthesis 2383–2392 (2010).
[17] Shirai, K., Yanagisawa, A., Takahashi, H., Fukunishi, K. and Matsuoka, M., "Synthesis and fluorescent properties of 2,5-diamino-3,6-dicyanopyrazine dyes," Dyes and Pigments 39, 49–68 (1998).
[18] Dorshow, R. B., Bugaj, J. E., Burleigh, B. D., Duncan, J. R., Johnson, M. A. and Jones, W. B., "Noninvasive fluorescence detection of hepatic and renal function," J. Biomed. Opt. 3, 340–345 (1998).
[19] Prueksaritanont, T., Lui, C. Y., Lee, M. G. and Chiou, W. L., "Renal and non-renal clearances of iothalamate," Biopharm. Drug Dispos. 7, 347–355 (1986).
[20] Yamaoka, T., Tabata, Y. and Ikada, Y., "Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice," J. Pharm. Sci. 83, 601–606 (1994).
[21] Roch-Ramel, F. and De Broe, M. E., [Renal handling of drugs and xenobiotics. In Clinical Nephrotoxins: Renal Injury from Drugs and Chemicals], Kluwer Academic Publishers, Dordrecht, 21–46 (2003).