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Molecular-Scale Diagnostics

Medication Detection by a Combinatorial Fluorescent Molecular Sensor**

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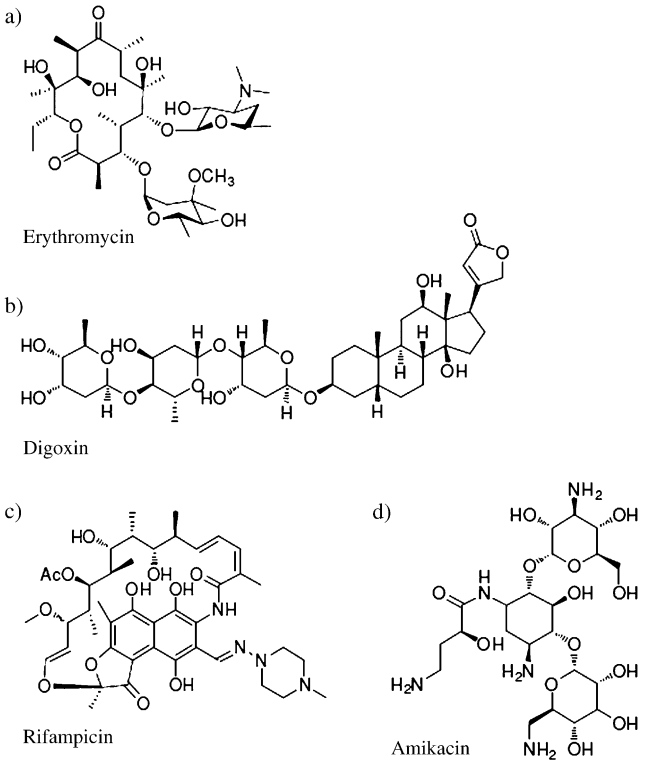
There is wide interest in the development of fluorescent molecular sensors triggered by several input signals and their application as computation and analytical devices.[1] Such sensors can imitate the function of electronic logic gates and circuits[2] and can also be applied to cellular imaging,[3] to clever chemosensing,[4] and as digital molecular tags.[5] The development of a "lab on a molecule"[6] for the simultaneous identification of Na^+, H^+, and Zn^{2+} highlights an exciting prospect for this field. Specifically, it shows the potential for the creation of molecular-scale analytical systems with high sensitivity[7] and high-throughput detection capabilities, akin to microarray sensing devices.

An inherent limitation of the use of these unimolecular fluorescent sensors[2–6] is the need to design a receptor for each target, which significantly limits their multiplicity. Therefore, an alternative class of "lab on a molecule" was developed in which the sensor–analyte interactions are monitored by an array of detection methods.[8,9] The efficiency of this approach was demonstrated with the selective detection of several metal ions by a sensor molecule bearing only one type of receptor.[8] This important advance,[10] however, involves the use of different instrumentations, which may complicate high-throughput analysis.

A third strategy that enables high-throughput, multianalyte sensing by individual molecules is combinatorial sensing.[11,12] By use of the "nose/tongue" approach,[13] a chromophoric ligand[11] and, more recently, a ^{13}C-labeled molecule[12] were cleverly designed to differentiate between metal ions and fullerenes, respectively. Although these systems do not operate in the fluorescence mode and so far cannot compete with the remarkable abilities of cross-reactive sensor arrays to discriminate between organic compounds and biomolecules,[14] they indicate the potential for the development of multianalyte fluorescent molecular sensors with superior analytical capabilities.

Herein, we describe the design and function of a combinatorial fluorescent sensor that takes molecular-scale diagnostics[3–12] a step further. The molecular analytical system presented herein combines several recognition elements as well as four emission channels and utilizes distinct photophysical processes that enable us to identify a wide range of pharmaceuticals and analyze drug concentrations and combinations in urine samples in a high-throughput manner.

The development of methods for the verification of drug content at point-of-care has been receiving growing international attention.[15] We therefore selected four drug families commonly associated with counterfeiting or medication errors as test cases for our molecular sensor (Scheme 1; see Figure S1 in the Supporting Information). Macrolides, aminoglycosides, and rifamycins are large families of antibiotics whose counterfeits are highly prevalent in the developing world.[16] Cardiac glycosides, used for treating heart conditions, have been associated with substandard medication in developed countries[15] and are often involved in medication errors owing to their narrow therapeutic window and adverse drug interactions.[17]



Scheme 1. Representative structures of the a) macrolide, b) cardiac glycoside, c) rifamycin, and d) aminoglycoside drug families.

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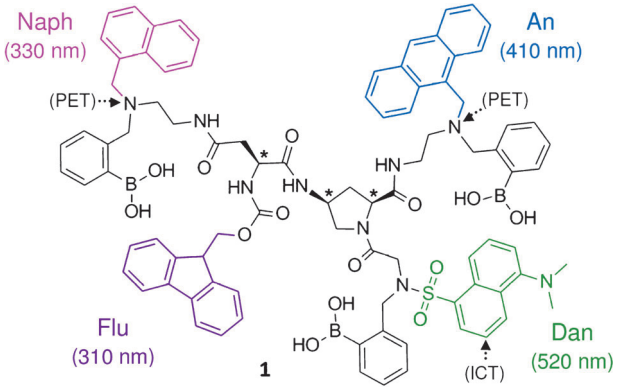
To be able to identify different macrolides, aminoglycosides, cardiac glycosides, and rifamycins with a single fluorescent molecule, we designed and synthesized sensor **1**, which combines hydrophobic recognition elements with boronic acid functionalities (Scheme 2; see the Supporting



Scheme 2. a) Structure of the monomolecular combinatorial sensor **1**. The emission wavelength of each dye, stereogenic centers, and the components involved in PET and ICT processes are indicated.

Information for synthetic details). The latter have been used extensively for the recognition of carbohydrates and their derivatives with individual fluorescent molecular sensors[18] or through colorimetric arrays.[14h–o] In **1**, three phenyl boronic acids form diol-binding receptors, while four spectrally overlapping fluorophores, namely, naphthalene (Naph), fluorenyl (Flu), anthracene (An), and dansyl (Dan), serve as hydrophobic units and as reporters that can transduce the binding event into a measurable optical signature. The attachment of these components to a *cis*-amino-L-proline scaffold provides a flexible and chiral molecular cavity that can accommodate and discriminate between the different medications. The binding of different analytes distinctly affects the emission of each dye by interfering with the photoinduced electron transfer (PET), internal charge transfer (ICT), and fluorescence resonance energy transfer (FRET) processes. The combination of these effects provides a vast number of unique optical signatures.

Figure 1 a shows the excitation and emission spectra of the individual fluorescent receptors, specifically, each boronic acid–dye pair (i.e., Naph*, An*, and Dan*) and a fluorenyl–aspartic acid derivative (Flu*). The emission spectra of Naph* and Flu* overlap with the excitation spectra of An* and Dan*. Therefore, illumination at 270 nm should result in an emission pattern ranging across the UV/Vis spectrum (Figure 1 b) owing to FRET between the donors (i.e., naphthalene and fluorenyl) and acceptors (i.e., dansyl and anthracene) as well as direct excitations, mainly of naphthalene, fluorenyl, and dansyl. An additional energy-transfer process that occurs to a lesser extent involves FRET between anthracene and dansyl. Because FRET largely depends on the distances between the donors and acceptors, conformational changes that occur upon drug binding is one factor that contributes to the generation of distinct fluorescence signatures.

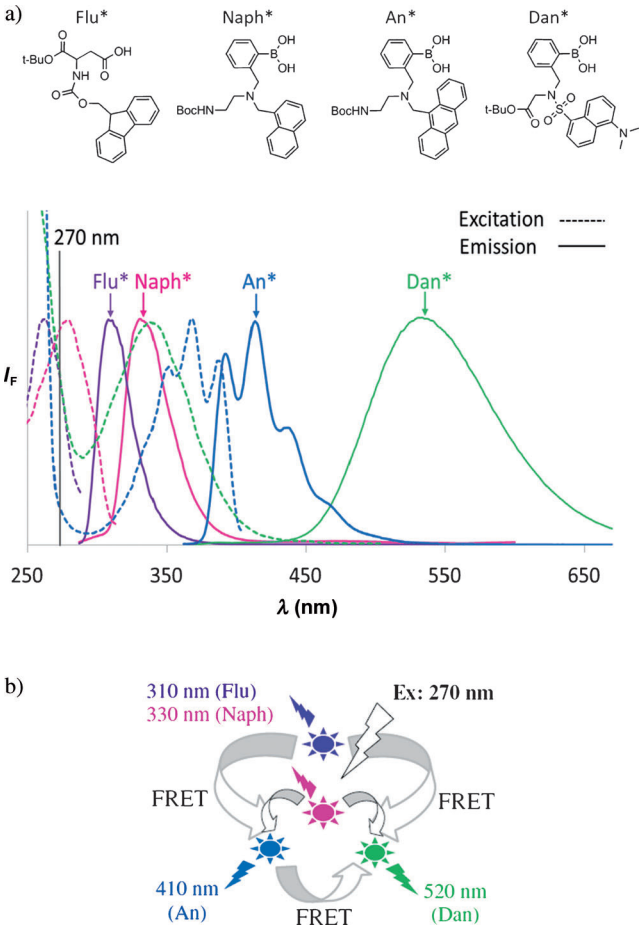


Figure 1. a) Normalized excitation and emission spectra of fluorenyl–aspartic acid (Flu*), naphthalene–boronic acid (Naph*), anthracene–boronic acid (An*), and dansyl–boronic acid (Dan*) derivatives dissolved in methanol. b) Schematic representation of the possible intramolecular FRET processes upon excitation at 270 nm. Boc = *tert*-butoxycarbonyl.

To be able to differentiate between drug molecules that may not inflict substantial structural changes, we incorporated two PET-based sensors,[18b] namely, naphthalene–boronic acid and the well-known anthracene–boronic acid,[19] into the structure of **1** (Scheme 2). In this way, the binding of a drug molecule to a boronic acid adjacent to a nitrogen atom and a fluorophore should directly trigger an increase in their emission intensity. Another outcome of PET is quenching of the emission of the naphthalene donor, which may disrupt the overall FRET processes required for operating the sensor. Therefore, we incorporated an additional fluorescence donor (i.e., a fluorenyl group) into the sensor to ensure efficient energy transfer to anthracene and dansyl, as well as to increase the variability of the optical patterns generated by the molecular device.

A third signaling mechanism that further contributes to the discrimination ability of the sensor is ICT.[7] Dansyl is an environmentally sensitive ICT probe commonly used to monitor supramolecular host–guest interactions. We reasoned that the binding of a guest molecule within the cavity[20] would

induce a change in its immediate environment, for example, by disrupting intramolecular hydrogen bonds or π-stacking.

The structure of **1** optimized by using density functional theory (DFT) supports the possibility of such intramolecular interactions (Figure 2). The modeling shows that the molecule

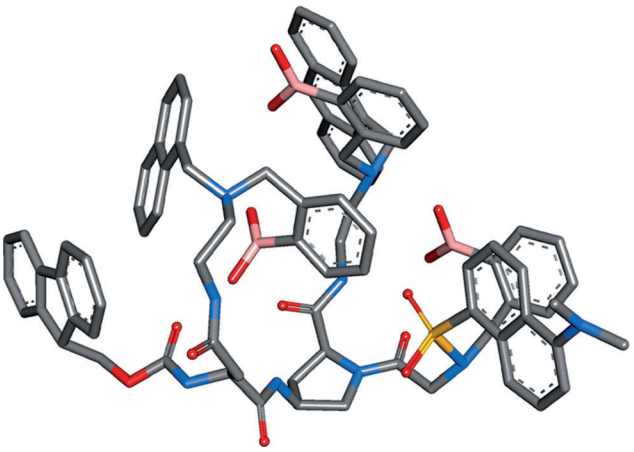


Figure 2. DFT-optimized structure of **1** (C gray, N blue, O red, B pink, S yellow; hydrogen atoms are omitted for clarity).

forms a flexible and structurally preorganized cavity in which the four dyes and three phenyl boronic acid groups project in the same direction (see the Supporting Information for computational details). Importantly, the structure is maintained through intramolecular hydrogen bonds and π-stacking. For example, the phenyl boronic acid next to the dansyl group forms π-interactions with the adjacent aromatic ring (Figure 2), whereas its hydroxy groups are hydrogen-bonded to an amide and an amine on the neighboring arm as well as to a nearby carbonyl group (see Figure S3 in the Supporting Information).

We tested the efficiency of the design by determining the ability of **1** to generate unique optical signatures for different drugs (Figure 3). Excitation of the sensor at 270 nm resulted in fluorescence emission across the UV/Vis spectrum, as expected from intramolecular FRET processes (Figure 1). The addition of cardiac drugs or antibiotics to a solution of **1** in methanol resulted in readily distinguishable changes in the different emission channels. Interestingly, whereas macrolides, aminoglycosides, and cardiac glycosides were detected at a 5 mM concentration, a much higher sensitivity for rifamycins (i.e., rifampicin, rifabutin) was observed, presumably as a result of additional π-interactions and quenching of fluorescence by their aromatic core. Thus, rifamycins can be identified at low micromolar concentrations (Figure 3, Figure 4).

Principal component analysis (PCA) was applied (see the Supporting Information) to distinguish between the emission patterns generated by the medications considered in this study (Figure 4, drugs **2**–**13**). These drugs are commonly involved in cases of forgery or poor-quality manufacturing. For example, in many developing countries, about 30% of the most essential antibiotics, such as erythromycin (**6**), roxithromycin (**7**), and rifampicin (**12**), may contain the wrong

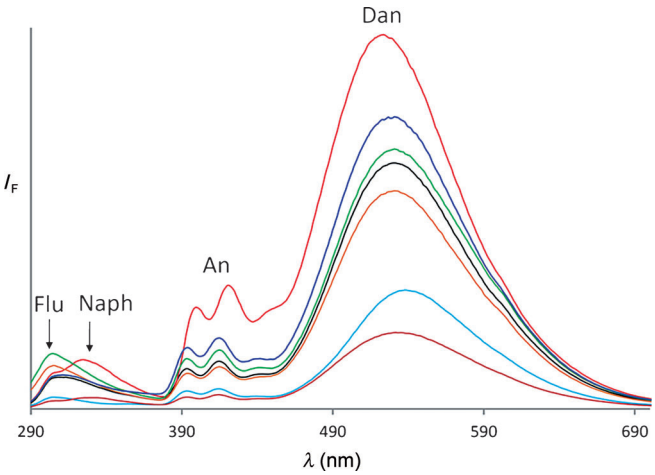


Figure 3. Representative emission signatures generated by **1** (black) upon the addition of 5 mM ouabain (red), 5 mM digitoxin (green), 5 mM digoxin (orange), 5 mM erythromycin (blue), 100 μM rifampicin (light blue), and 100 μM rifabutin (brown). Excitation wavelength: 270 nm.

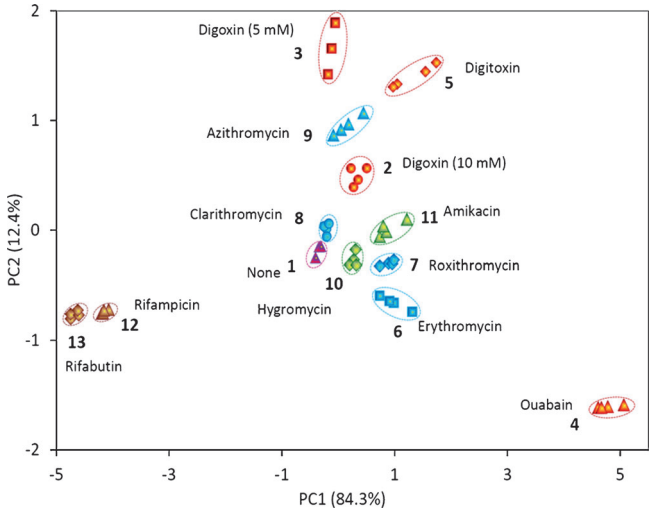


Figure 4. PCA mapping of emission patterns generated by the cardiac glycosides (**3**–**5**, red), macrolides (**6**–**9**, blue), and aminoglycosides (**10** and **11**, green) at a concentration of 5 mM, by the rifamycins (**12** and **13**, brown) at a concentration of 100 μM, and by a double amount of digoxin (**2**, red).

ingredients (e.g., erythromycin instead of roxithromycin[21]), no active ingredients, or incorrect doses.[16] Similarly, the recent discovery of incorrect doses of digoxin (**2**, **3**) in tablets of the drug in the USA led to an urgent recall of the drug nationwide.[15] Drugs randomly selected from the training set could be identified by our sensor with 93% accuracy.

Beyond the authentication of commercially available drugs, analytical systems that can detect various drug concentrations and combinations play an important role in pharmacokinetic studies. For example, the parallel analysis of rifampicin and D-xylose levels in urine is used to diagnose rifampicin malabsorption in HIV patients with tuberculosis.[22] In such studies, patients are given a normal dose of rifampicin and a much larger amount of the saccharide.[23]

To demonstrate the potential of combinatorial molecular sensors to distinguish between various drug concentrations and combinations within medicinally relevant samples, we subjected **1** to human urine loaded with different ratios of D-xylose and rifampicin. Samples with just rifampicin (sample A) or D-xylose (sample F) induced distinct changes in the emission patterns (Figure 5a). Notably, the addition of sample A resulted in a substantial decrease in anthracene and dansyl fluorescence, whereas the addition of sample F mainly led to an enhancement in their emissions (Figure S2). Similar measurements were performed with urine samples containing different antibiotic/saccharide ratios, and PCA was used to differentiate between the patterns generated at the four emission channels in which the maximal intensity changes were observed (Figure 5b). The molecular diagnostic system was able to identify unknown urine–drug samples with a 97% success rate.

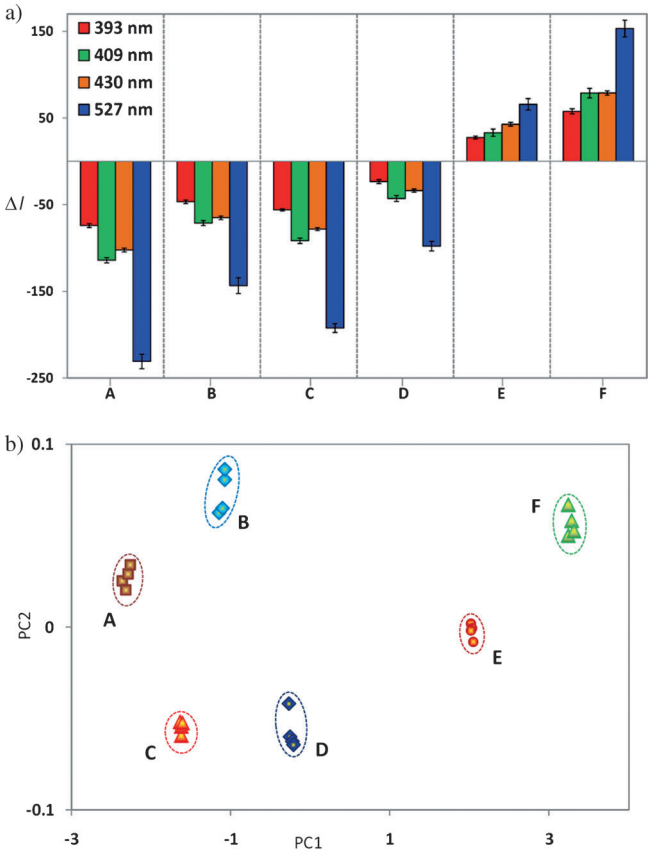


Figure 5. a) Fluorescence response (ΔI) of **1** to human urine containing: A) rifampicin (80 μg mL^{-1}); B) rifampicin (40 μg mL^{-1}); C) rifampicin (80 μg mL^{-1}) and D-xylose (30 mg mL^{-1}); D) rifampicin (40 μg mL^{-1}) and D-xylose (30 mg mL^{-1}); E) D-xylose (30 mg mL^{-1}); F) D-xylose (60 mg mL^{-1}); error bars correspond to the standard deviation. b) Corresponding PCA plot. Excitation wavelength: 345 nm.

To date, multianalyte sensing by individual molecules has mainly been focused on the detection of metal ions and protons. The ability of **1** to discriminate between various medications, as well as between analyte combinations in urine samples, demonstrates the potential for the application of such sensors in pharmacokinetics, therapeutic drug monitoring, and medicinal diagnostics. Moreover, it shows the feasibility of developing fluorescent molecular sensors with remarkable analytical abilities. The combinatorial sensor described herein, for example, is extremely simple to operate, since it avoids the need for device integration and it utilizes a single instrumentation, a single excitation wavelength, and a single incubation step, all of which enable straightforward analysis. We expect this study to contribute to the development of information processing at the molecular level[1,2] and, in particular, to multianalyte, molecular-scale diagnostics.[3–12]

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