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Steroid sensor

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Bulk aggregation based fluorescence turn-on sensors for selective detection of progesterone in aqueous solution **

*Seong Cheol Hong, Dhiraj P. Murale, Minju Lee, Seung Mi Lee, Joong Shin Park, Jun-Seok Lee**

Abstract: *Steroids are polycyclic compounds that share tetracyclic ring as core scaffold, and selective detection of a steroid is challenging due to their structural similarities. Here, we describe a discovery of chemosensors that recognize progesterone by alteration of self-aggregation state, and showed significant fluorescence turn-on. In this work, we screened self-aggregated 48-membered dansyl library against series of metabolites in aqueous buffer and discovered two compounds (**PG-1**, **PG-2**) exhibited exceptional selectivity for progesterone. Following studies of aggregation properties of probes using dynamic light scattering and transmission electron microscopy supports progesterone recognition lead to the generation of bulk aggregates that induce fluorescence enhancement. Though many fluorescence sensing mechanisms have been proposed, sensing mode based on the bulk aggregate formation of fluorophore has never been reported, and we believe it will open a new avenue of chemosensor design.*

Steroids are one of the major classes of hormone along with the eicosanoids and peptides. Each steroid metabolite plays a role as chemical messengers that modulate essential physiological responses including gene transcription, endocrine production, and humoral immunity.[1] Considering their complexity of biological signaling functions, disorder of the steroid metabolism cause series of diseases, especially related to the early development of fertility and progression of pregnancy.[2] Additionally, the administration of exogenous steroids induces physical responses including muscle growth and blood cell production increment.[3] Therefore, the straightforward detection method for individual steroid metabolite is crucial for diagnosis of the disease and a doping test.

Selective detection of steroids, however, is extremely challengin due to the high similarity of their structures. Most steroids share tetracyclic hydrocarbon ring as core structure called gonan (cyclopentapherhydrophenanthrene), and possess different functiona groups at distinct positions. Nature has developed steroid receptor through evolution and orchestrated individual steroid specifi signaling,[1b] but there is no such selective recognition metho available for steroid sensing *in vitro* yet. The current gold standar method of steroid identification is metabolite profiling based on th gas/or liquid chromatography coupled with mass spectrometry (GC MS, LC-MS),[4] which is now possible to identify and quantitat hundreds of steroids in a single chromatography injection.[5] Thoug it provides comprehensive data, it has drawbacks in that the analys routine is time-consuming and requires sophisticated machine as we as specialized skills for sample preparation procedures. Besides, othe analytical platforms have been exploited to detect steroids, fo instance, electrochemical sensors,[6] fluorescence protein sensors,[or enzyme-based biosensors.[8] To achieve selective recognition of steroid, these platforms take advantage of antibody or aptamer, but is also well-known that successful developments of antibody o aptamer for a specific small molecule without cross-reactivity ar awfully rare.[9]

Comparing to the conventional steroid sensing technique fluorescent chemosensor has merits, such as simple and fa detections, no requirements of a special facility, and high cos efficiency. Fluorescent sensors are organic fluorophore that recogniz the target molecule and eventually change their fluorescence emissio intensity or color. Key-and-lock concept based synthetic receptor has been most extensively exploited to design fluorescence senso rationally, and majorities of their mechanisms of fluorescenc response were proposed as photo-induced electron transfer (PET),[1 or molecular rigidity-induced enhancement.[11] By far, cyclodextrin the leading recognition host for steroids,[12] but selectivity contr against single steroid metabolite was not accomplished yet.[13]

Recently, another mode of fluorescence turn-on mechanism ha proposed that is a disaggregation of organic fluorophores.[14] In th disaggregation sensor, fluorescence was quenched by self-assemble structure, and fluorescence turn-on signal prompted by targ recognition-driven disassembly. Although these disaggregatio sensors have been designed by highly ordered template-aggregate and selective ligand-receptor interactions for limited targets,[14b, 15] the investigations of fluorescence sensing for steroid by aggregation or disaggregation phenomena has never been investigated. Here, we report a discovery of fluorescence sensors for progesterone, in which fluorescence turn-on was induced by alteration of fluorophore aggregation.

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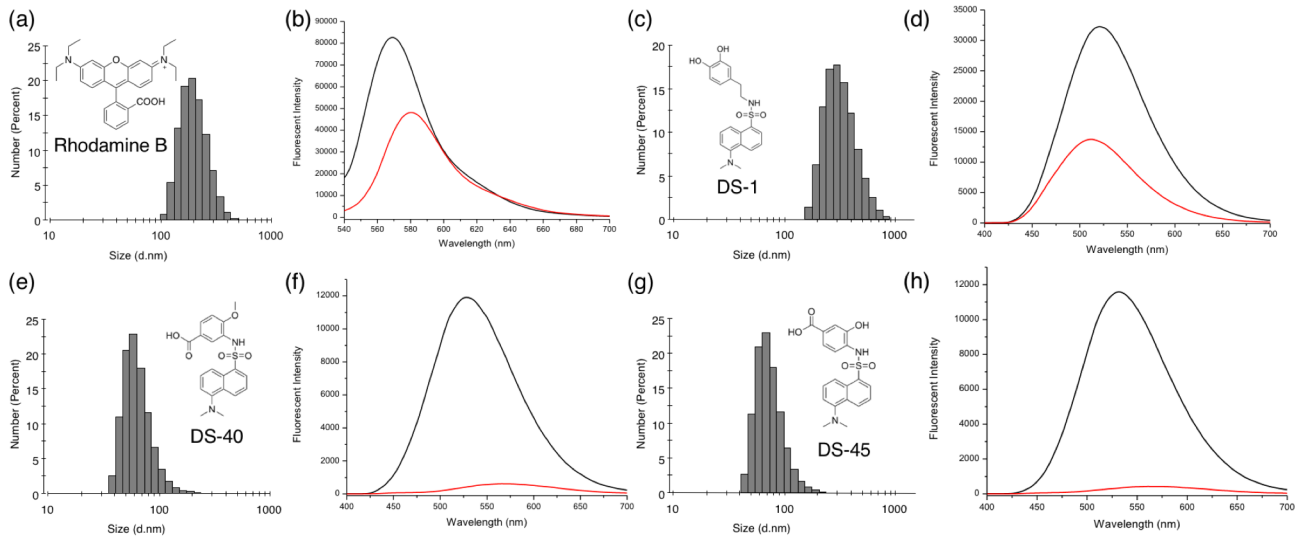

Figure 1. Dynamic light scattering (DLS) signal of self-aggregates in PBS 7.4 buffer (a, c, e, g) and fluorescence emission spectra (b, d, f, h) in 100 % ethanol (black line) and 5 % ethanol in PBS pH7.4 buffer (red line). In ethanol, all fluorophore does not give DLS signals, but they showed sharp signals in aqueous condition. Rhodamine B (a, b), **DS-1** (c,d), **DS-40** (e,f), **DS-45** (g, h).

We recently synthesized a 48 membered combinatorial library of dansyl fluorophores (**DS** library) and demonstrated their usage for pH sensors and live cell imaging agents.[16] In our following study, we observed most of dansyl fluorophores showed consistently quenched fluorescence in aqueous media compare to polar organic solvent (Table S1). Since few fluorophore scaffold has been known to form a self-assembled aggregate in aqueous media due to their hydrophobicity, we suspected dansyl fluorophore might have similar self-aggregation which inducing quenching effect in water due to their neutral and hydrophobic properties. We initially checked the aggregate formation of **DS** library and other representative fluorophores (rhodamine B, and fluorescein) using dynamic light scattering (DLS) in ethanol and an aqueous solution (Figure 1). Neutral **DS** compounds showed apparent aggregation ranges in 20 – 300 nm diameter size depending on their structures only in aqueous condition, and their fluorescence was also quenched accordingly. Rhodamine B also forms 200 nm size aggregates possibly due to their hydrophobic characteristic even though their positive charge, but negatively charged fluorescein does not form aggregates. Interestingly, strong fluorescence quenching was observed for small particle forming compounds (**DS-40**, **DS-45**) compared to the bigger particle forming compounds (rhodamine B, **DS-1**). Because restrained fluorescence quantum yield is a favorable photophysical property of diversity-oriented fluorescence sensor discovery strategy,[17] we envisaged our **DS** library is suitable candidates for turn-on sensor for various metabolites in aqueous, which is possibly altered by aggregation/disaggregation states.

To systematically explore fluorescence responses of **DS** library, we screened their fluorescence emission response profile against 29 kinds of metabolites, including amino acids, oxidoredox-related molecules, and steroids (Figure 2). Each target molecule was tested at four concentration ranges by mixing with the 25 μM **DS** compounds to determine dose-dependent intensity changes in the effective concentration (48 DS probes x 29 analytes x 4 doses = 5,568 primary screening assay). Fluorescence spectra were measured using fluorescence microplate reader, and fluorescence intensities at the maximum emission wavelength (λ_{max}) for each probe were used to calculated fold change. Average fold change value of fluorescence emissions between with and without analytes were 0.93 that indicates most **DS** compounds does not show prominent fluorescence responses. We found several **DS** compounds exhibited fluorescence intensity increments for steroids despite their relatively lo concentration compared to other analytes. Interestingly, many those steroid responsive **DS** compounds (**DS-30**, **DS-32**, **DS-33**, **DS 38**, **DS-43**, **DS-46**) contains electron withdrawing groups in th benzene ring (Figure S1).

Primary hit compounds that exhibited fluorescence turn-on in th presence of any steroids were re-evaluated to confirm the primar profiles, and 2 **DS** compounds (**DS-43**, **DS-46**; Figure 3-a) reveale remarkable selectivity against progesterone among 5 steroic (cholesterol (CHO), testosterone (TES), progesterone (PGT), 17-β estradiol (EST), dehydroisoandrosterone (DHEA); Figure 3-b) in th primary screening results. Encouraged by such high selectivity, w further examined fluorescence response of the two hit compounc against additional 8 steroids (total 13 steroids) including progesterone derivative, 17-α-hydroxyprogesterone (17-α -OH PGT), in which the structure only differs an additional hydroxy group at 17 position of gonane. From the comparison data, both h

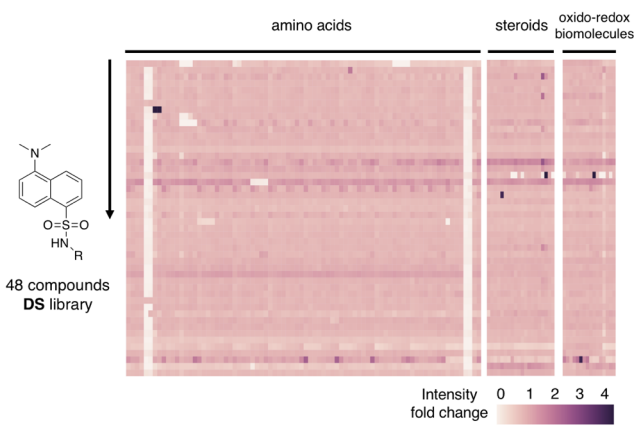

Figure 2. Heatmap diagram of primary screening of **DS** library for 29 metabolites. Fold change values were calculated based on fluorescence emission change of each **DS** compound (25 μM) upon mixing with target metabolites (amino acids: 1 mM, 100 μM, 1 μM, 10 nM, oxido-redox biomolecules: 1 mM, 100 μM, 1 μM, 10 nM, steroids: 10 μM, 1 μM, 10 nM, 0.1 nM). Full list of metabolites and assay conditions in SI.

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compounds showed outstanding selectivity to progesterone. **DS-43** and **DS-46** exhibited fluorescence enhancement to progesterone up to 15.3- and 25.4-folds, respectively (Figure 3-c). Compared to the other kinds of steroids, the most cross-reactive species was 17- α -OH-PGT for **DS-46** that was about 2-fold emission increase. Based on the selectivity and color, we dubbed our **DS-43** as Progesterone Green-1 (**PG-1**), and **DS-46** as Progesterone Green-2 (**PG-2**).

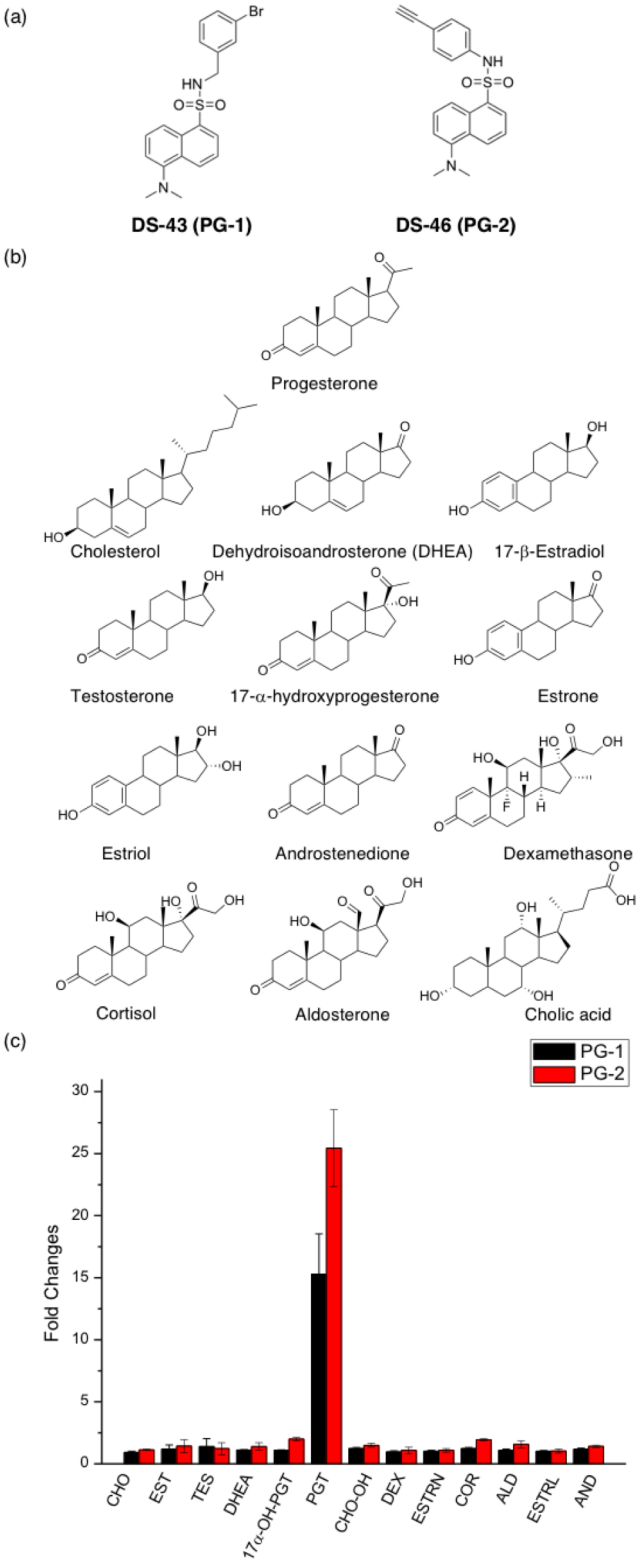

Figure 3. (a) Structure of Progesterone Green (**PG-1**, **PG-2**), (b) chemical structures of steroids, (c) bar graph of fluorescence intensity fold change upon addition of 13 steroids. 10 μM of **PG-1** and **PG-2** compounds were mixed with series of steroids (100 μM) in 5% ethanol PBS 7.4 buffer solution. Fluorescence emission spectra were obtained by excitation at 365 nm after 5 min incubation, and intensity fold change were calculated from the emission maximum wavelength ($\lambda^{PG\text{-}1}_{max}$ = 490 nm, $\lambda^{PG\text{-}2}_{max}$ = 500 nm).

Next, we checked photophysical property changes upon addition of PGT to **PG-1** and **PG-2** (**PG**s). Dansyl scaffold has been known to have two excitations; one around 260 nm for locally excited (LE) planar state which configuration between dimethyl amino and naphthalene π-conjugation system is planer, and the other at 330 nm for twisted intramolecular charge transfer (TICT) state, which th structure is orthogonally aligned.[18] Series of the concentration c PGT (0 to 100 μM) was mixed with individual chemosensor (10 μM incubated for 5 min, and measured excitation (λ_{ex}), emission (λ_{em} profile (Figure S2, S3, S4). Both **PG**s showed significar bathochromic shift for LE (**PG-1**: 260 to 270 nm; **PG-2**: 265 to 27 nm) and TICT (**PG-1**: 335 to 350 nm shift; **PG-2**: 330 to 355 nm shift) peaks in excitation spectra, and apparent emission intensit increments with emission maximum shift to short wavelength (**PG-1** 550 to 490 nm, 60 nm blue shift; **PG-2**: 540 to 500 nm, 40 nm blu shift). From the fluorescence enhancement, we calculated th detection limits of **PG-1** (1.21 μM) and **PG-2** (38.1 nM) in PBS a described in the previous report (Figure S5; 2.28 μM for **PG-1**, 4.5 μM for **PG-2** in blood spike-in condition).[19] Despite this level c detection (**PG-2**: 11.9 ng/ml) is slightly higher than the conventiona electrochemical methods (3.56 ng/ml), **PG-2** is the first fluorescenc sensor that can distinguish different steroids and we believe th fluorescence sensor based method has its unique merits for simple an fast detection.

To evaluate our hypothesis of sensing mechanism b assembly/disassembly property of **DS** library, we investigate aggregation status using various techniques. Aggregations of **PG** were examined with subsequent addition of PGT, DHEA, and 17-α OH-PGT. Solutions were prepared 5 % EtOH in PBS 7.4 buffer, an 100 μM of steroids were dissolved in the solution. In this condition steroids were fully dissolved, and we could not observe any signal i DLS. On the other hand, DLS analysis showed **PG**s form aggregate in the solution (Figure 4-a, b; **PG-1**: 37.9 nm, **PG-2**: 57.5 nm). To ou surprise, both **PG-1** and **PG-2** showed significant DLS signal shift t much bigger particle size upon addition of PGT within 5 min (Figur 4-c, d; **PG-1**: 157.1 nm, **PG-2**: 188.3 nm). It is noteworthy tha aggregation size shift was only observed for PGT same as the fluorescence response pattern (Figure 4-e, f, g, h). The aggregatio particle size was increased dose dependent manner (Figure S10), an longer incubation of **PG** probes and PGT exhibited size growin pattern of aggregate (Figure S11). In our bulk-aggregate formatio condition (10 μM PG probe, 100 μM PGT), the growing wa saturated approximately 2 hour for **PG-1** and 4 hour for **PG-2**. Sinc unique bulk-aggregate was formed within 5 min, which can be clearl distinguishable to the self-aggregates of **PG**s, we focused on clinica assay in this condition. Transmission electron microscope (TEM image also showed the clear formation of self-aggregates of **PG-1** and **PG-2** with a size that ranges from 30 to 60 nm in diameter (Figure 4-i, k, S12-a, S13-a), and the aggregation particle size increased upon addition of PGT up to 200 nm in diameter ranges (Figure 4-j, l, S12-b, S13-b). Like the DLS analysis, TEM images also exhibited dose dependent aggregate size increment pattern, and longer incubation time yield growing size of bulk-aggregates (Figure S14). Correlation between fluorescence turn-on and aggregation of probes were additionally examined by pair-wise comparison of data from organic solvent conditions, in which **DS** probes are fully dissolved. The higher percentage of ethanol in PBS buffer induces stronger fluorescence of **PG** probes themselves (Figure S6, S7). Besides, both **PG-1** and **PG-2** showed negligible fluorescence enhancement upon

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addition of PGT in ethanol solution (Figures S8, S9), and there was no DLS signals in ethanol solvent conditions.

These observations suggest that selective fluorescence turn-on phenomenon was occurred based on two reasons. The first one is the self-assembly induced quenching effect of the fluorophore in aqueous solution. Neutral, hydrophobic **DS** probes form self-aggregates and exhibit low fluorescence as a baseline in PBS buffer condition, but they are fully dissolved and show high emission intensity in organic solvents, which makes high background signal. Hence, quenched fluorescence state has more chance to turn-on depending on the micro-environmental changes. The second reason is a bulk aggregation formation by interaction with PGT. In aqueous conditions, self-aggregates of **PG**s could have better chance to interact with steroids due to their hydrophobic properties. From our diversity-oriented library screening data, it is clear that all steroids cannot induce a bulk aggregate formation, but PGT made an exceptional interaction with self-aggregates of **PG**s possibly due to their structural uniqueness and produced higher order structures. Although the detail molecular interaction between PGT and aggregates of **PG**s are not clear yet, to best of our knowledge, sensing mechanism based on the bulk aggregate formation of small molecule fluorophore has never been reported before. To further evaluate clinical application of our **PG**s for the progesterone detection, we tested our compounds for progesterone detection in pregnant women samples. Progesterone levels in blood and amniotic fluids are spontaneously increased during pregnancy, and perturbation o progesterone level could lead series of obstetric disorders.[20] W collected blood and amniotic fluid sample from two pregnant wome (more than 17 weeks of gestation), and tested **PG** probes signal wit non-pregnant control sample (Figure 4-m, 4-n, S15). From triplicate

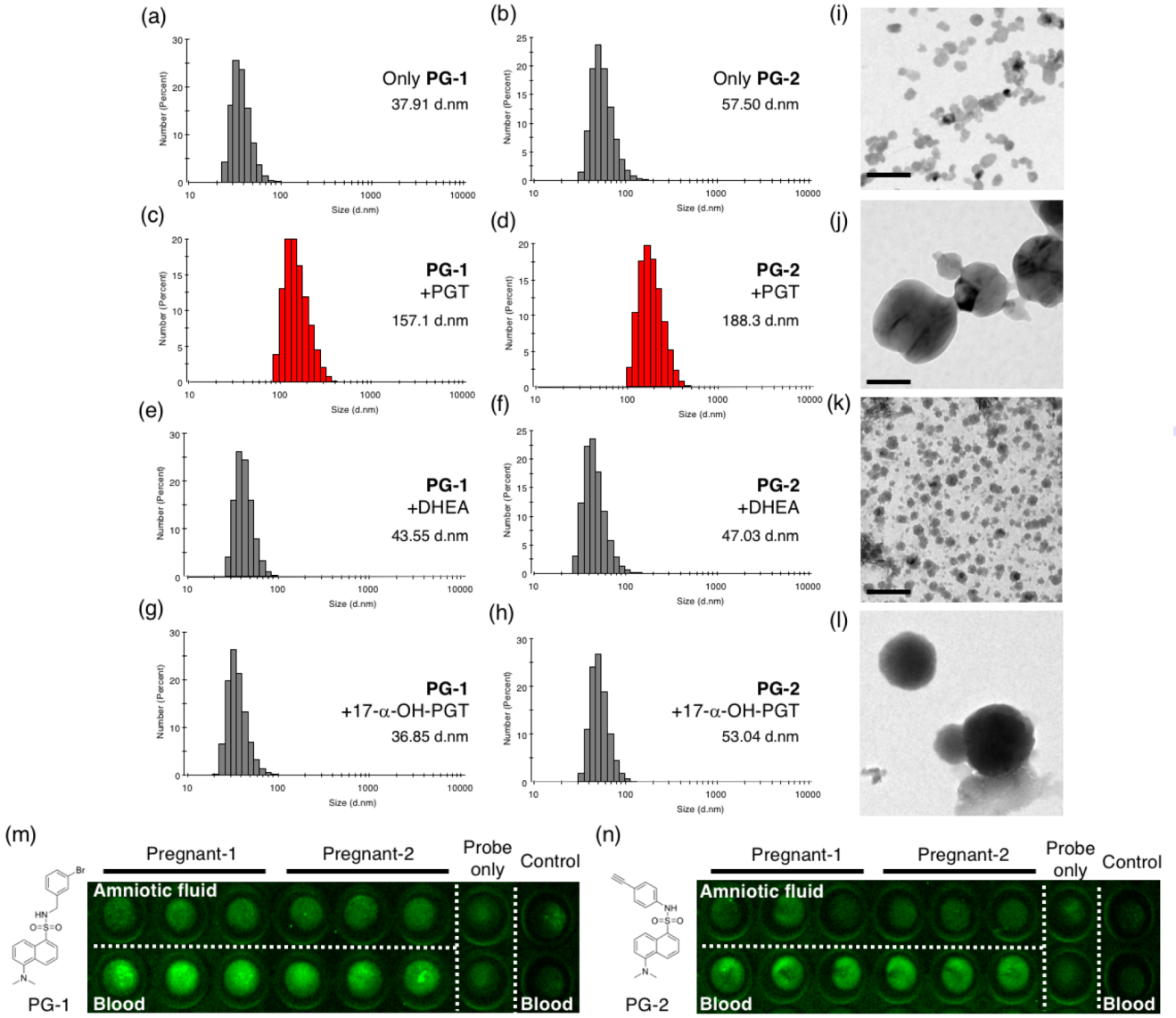

Figure 4. Microscopic and spectroscopic characterization of aggregate of **PG-1** and **PG-2**. (a-h) DLS analysis of particle size distributions of 10 μM **PG** probes only (a, b), 10 μM **PG** probes mixed with 100 μM progesterone (PGT) (c,d), 10 μM **PG** probe together with 100 μM dihydroisoandrosterone (DHEA) (e,f), 10 μM **PG** probe mixed with 17-α-hydroxyprogesterone (17-α-OH-PGT) (g,h). (i-l) TEM image of self-aggregates of **PG-1** (10 μM) (i), aggregates of **PG-1** (10 μM) together with progesterone (100 μM) (j), self-aggregates of **PG-2** (10 μM) (k), aggregates of **PG-2** (10 μM) mixed with progesterone (100 μM) (l). (m) Fluorescence scanner image of **PG-1** mixed with clinical samples (amniotic fluids and blood samples from two pregnant women were compared with control blood sample). (n) Fluorescence scanner image of **PG-2** mixed with same set of clinical samples as (m). All experiments were performed in 5% EtOH containing PBS 7.4 buffer. Scale bar is 200 nm.

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experiment, our primary results proved both **PG-1** and **PG-2** compounds can differentiate pregnant women samples out of control. From the results, amniotic fluids showed less turn-on effect than blood sample possibly due to the distinct ingredients of bio-fluid, but our data demonstrated the first fluorescent chemosensor based progesterone monitoring assay in clinical samples.

In conclusions, we have developed fluorescence sensors (**PG-1**, **PG-2**) for progesterone that can selectively form bulk aggregate together with fluorescence turn-on in aqueous solution, and demonstrated their progesterone monitoring application in clinical samples. DLS and TEM data supported progesterone selective bulk aggregate formation, which is over hundred nanometer size, from self-aggregates of the probe that is approximately tens of nanometer size. Since both LE and TICT excitations were observed from self-aggregates and bulk aggregates with progesterone, **PG-1** and **PG-2** does not produce a highly-ordered aggregation, but rather stochastically mixed structures. Though molecular recognition event of lock-and-key mechanism has been proposed as a major driving force to produce fluorescence signal, several recent reports suggest aggregation/disaggregation status of organic fluorophore might be the mechanism of a turn-on.[14] Our results suggest that the role of aggregation /disaggregation should be carefully examined for organic chemosensor developments. More detail molecular interaction between progesterone and **PG** probes are under investigation.

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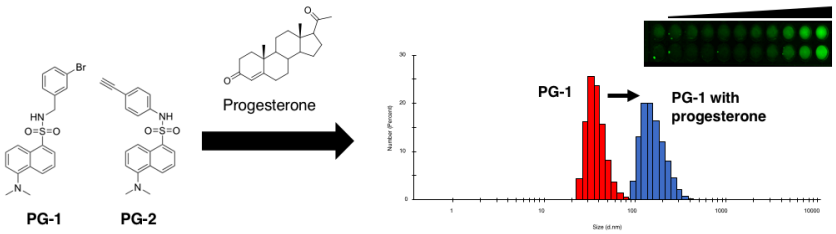
Steroid sensor

Seong Cheol Hong, Dhiraj P. Murale, Minju Lee, Seung Mi Lee, Joong Shin Park, Jun-Seok Lee* _____ **Page – Page**

Bulk aggregation based fluorescence turn-on sensors for selective de-tection of progesterone in aqueous solution



A fluorescent library based on dansyl fluorophore were screened for variou metabolite. We discovered two probes (PG-1, PG-2) showed exceptional selectivi for progesterone with more than 15-fold enhancement of fluorescence. Finally, w unveiled the fluorescent enhancement is induced by bulk aggregation progesterone together with PG-1 and PG-2.