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ABSTRACT

Currently, semiconductor nanoparticles known as quantum dots (QDs) have attracted interest in various application fields such as those requiring sensing properties, binding assays, and cellular imaging and are the very important in the acceleration of drug discovery due to their unique photophysical properties. Here, we applied graphene quantum dots (GQDs) for the binding assay of membrane progesterone receptor alpha (mPRα), one of the probable membrane receptors that have potential in drug discovery applications. By coupling the amino groups of mPRα with GQDs, we prepared fluorogenic GQD-conjugated mPRα (GQD-mPRα). When mixed with a progesterone-BSA-fluorescein isothiocyanate conjugate (P4-BSA-FITC) to check the ligand receptor binding activity of GQD-mPRα, fluorescence at 520 nm appeared. The fluorescence at 520 nm was reduced by the addition of free progesterone into the reaction mixture. GQD-coupled BSA (GQD-BSA) did not show a reduction in fluorescence at 520 nm. The results demonstrated the formation of a complex of GQD-mPRα and P4-BSA-FITC with ligand receptor binding. We established a ligand binding assay for membrane steroid receptors that is applicable for high-throughput assays.

Keywords: graphene quantum dots, membrane progesterone receptor, progesterone, FRET, steroids

1. Introduction

A group of steroid hormones, progestins, have a variety of functions in the regulation of the reproductive function of the body [1,2]. The natural progestin progesterone plays an important role in regulating egg maturation and the menstrual cycle along with the sex steroid estrogen in humans [3]. Synthetic progestin imitates the effect of progesterone and controls reproductive tissues in female and male vertebrates. Thus, progestins have been applied as fertility control treatments, contraceptives, and anticancer drugs [4]. More recently, progestins have attracted attention for Alzheimer's hormone therapy [5]. Currently, it is becoming clear that progestins have some nongenomic effects mediated by membrane progestin receptors (mPRs) on plasma and induce rapid intracellular changes [6,7].

mPRs are members of a group of cell surface receptors, i.e., the progestin and the AdipoQ receptor (PAQR) family [8]. The receptor containing the seventh gene of the PAQR family, mPRα (PAQR7), is one of the protein subtypes that have been suggested to be involved in physiological roles in reproductive tissues [6]. Although the 3D structure of PAQR7 has still not been determined, computer modeling predicts that the proteins have seven typical transmembrane domains as G protein coupled receptors (GPCRs) [9,10].

Many mPR-mediated nongenomic actions of progestins have been demonstrated, such as induction of oocyte maturation [11,12], quick activation signaling of breast cancer cells [7], mammalian sperm hypermotility [13], and induction of lordosis (female mating acceptance behavior) [14]. The action of neurosteroids in the brain is of particular interest. A progestin in the brain, allopregnanolone, is attracting attention as a therapeutic drug for Alzheimer's disease [5]. Recently, it was suggested that the antiapoptotic actions of allopregnanolone on neuronal cells are mediated through mPRs [15]. Additionally, it was

demonstrated that allopregnanolone promotes the development of chick Purkinje cells via mPR [16].

The mPR molecule is also drawing attention in relation to cancer. Cancer-related reports include high expression levels of mPR molecules in breast cancer, ovarian cancer and brain cancer [7]. Thus, it is suggested that the mPR molecule should be used as a marker for cancer diagnosis [17]. A notable report on the action of progesterone on cancer cells showed that progesterone suppresses cancer infiltration, and it has been shown that this effect is transmitted via mPR [18].

Quantum dots (QDs) are very small semiconductor particles a few nanometers in size that have optical and electronic properties that differ from those of larger particles due to quantum mechanics [19]. When QDs are illuminated by UV light, an electron in the quantum dot can be excited to a state of higher energy and release its energy by the emission of light. The color of the light depends on the energy difference between the conductance band and the valence band of QDs.

One application of quantum dots in biology is as donor fluorophores in Förster resonance energy transfer (FRET), where the large extinction coefficient and spectral purity of these fluorophores make them superior to molecular fluorophores [20]. It is also worth noting that the broad absorbance of QDs allows for the selective excitation of the QD donor and minimum excitation of a dye acceptor in FRET-based studies [21]. The applicability of the FRET model, which assumes that the quantum dot can be approximated as a point dipole, has recently been demonstrated [22].

As a class of zero-dimensional nanomaterials, graphene quantum dots (GQDs) have been discovered recently. GQDs along with their doped or functionalized nanocomposites have emerged as the most promising nanomaterials in several fields because of their

extraordinary optical, thermal, and electronic properties, such as electronics [23,24], energy storage [25], catalysis [26], materials science [27] and biomedical engineering [28]. More recently, fluorescence assays using fluorophores or transition metal chalcogenide-based QDs have become significant for achieving high sensitivity and rapid response to metal ion and organic detection [29,30].

Previously, we succeeded in the production and purification of recombinant human mPRα with progesterone binding activity by using a yeast protein expression system [31]. Since the binding assay system for mPRα is expected to be applied for screening new compounds for pharmaceuticals, we attempted to establish a new assay system using recombinant human mPRα protein and GQDs in this study. Here, we produced soluble GQD-mPRα and established a new steroid binding assay with fluorescently labeled progesterone.

2. Materials and methods

Materials

Citric acid, N-(3-Dimethylaminoprpyl)-N`-ethylcarbodiimide hydrochloride, N-hydroxysuccininide and steroids (corticosterone, cortisol, progesterone, testosterone, 17β-estradiol, 17α-methyl-testosterone, mifepristone) were purchased from Sigma Aldrich Chemicals (St. Louis, MO). 17α-hydroxyprogesterone and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Spectra 3.5 kDa dialysis membranes were purchased from Spectra Laboratories Inc. (USA).

Preparation of hmPRα

Human mPRα (hmPRα) proteins were expressed and purified as previously described [31].

Preparation of GQDs

The GQDs were prepared by using direct pyrolyzation of citric acid [32]. In a typical procedure of GQD preparation, 2 g of citric acid was added into a 100 mL round-bottom flask and heated to 200 °C by using a drying oven. The solid crystal citric acid became liquid and turned yellowish after 5 min. Then, an orange color appeared after heating for 20 min, indicating the formation of GQDs. In addition, different reaction times ranging from 20 to 45 min were examined, and a reaction time of 25 min was chosen in this study to fabricate the as-prepared GQDs with a high quantum yield (QY). Excess heating was strictly avoided because overheating may possibly cause the formation of graphene oxides. The obtained orange liquid containing GQDs was then added dropwise into 100 mL of 10 g/L sodium hydroxide (NaOH) solution under vigorous stirring. After neutralization to pH 7.0 with NaOH, an aqueous solution of GQDs was obtained and preserved in the dark at 4 °C for further use. The QY of prepared GQDs was determined by using fluorescein as the standard solution (Φ=0.79). The QY was calculated to be 0.124, which was same as the original report [32].

*Preparation of GQDs coupled mPR*α

The GQD-labeled mPRα (GQD-mPRα) was prepared by coupling the amine group/N-terminus of mPR with the carboxylic acid group of GQDs using the standard N-ethyl-N'-(3-(dimethylamino) propyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) reaction at room

temperature at pH 6.0 (Fig. 1A) [33]. As a control, GQD-labeled BSA (GQD-BSA) was prepared in the same manner as GQD-mPRα. Briefly, EDC and NHS were added to the GQD solution under vigorous stirring (on a small scale: 3 mg of EDC and 4 mg of NHS for 10 mL of GQD solution, on a large scale: 20 mg of EDC and 25 mg of NHS for 60 mL of GQD solution). Then, mPRα or BSA solution (on a small scale: 100 μL of 0.43 mg/mL mPRα solution or 0.5 mg/mL BSA solution, on a large scale: 60 mL of 0.14 mg/mL mPRα solution) was added to the GQD solution, and the reaction was performed for 2 h under vigorous stirring at room temperature. Then, the final solution was dialyzed in a 3.5 kDa dialysis bag for 24 h (dialysate was replaced with DDW every 8 h) to remove the unreacted chemicals. Prepared GQD-mPRα and GQD-BSA were stored at -30 °C.

Preparation of FITC-labeled progesterone-coupled BSA (P4-BSA-FITC)

P4-BSA-FITC was prepared by a standard reaction for FITC labeling of progesterone-coupled BSA (4-Pregnen-3,20-Dione 3-O-Carboxymethyloxime/BSA, Steraloids Inc. USA).

Binding assay using mPRα-GQDs and P4-BSA-FITC

Except for the test chemicals, mPRα-GQDs and P4-BSA-FITC were mixed in a reaction mixture of phosphate buffer, pH 7.4. To a well of a 96-well plate, 2 μl of test compound dissolved in ethanol was applied. Then, 98 μl of reaction mixture (final concentrations of mPRα-GQDs at 9 μg/ml mPRα, P4-BSA-FITC at 14 μg/ml BSA) was added and mixed (Fig. 1B). The prepared reaction mixture was incubated in the dark for 2 hrs at room temperature. Then, the fluorescence intensity of each well was measured by a

fluorescence microplate reader (Varioskan^{TM} LUX, Thermo Scientific, Waltham, USA) at an excitation wavelength of 370 nm and an emission wavelength of 520 nm.

3. Results

3.1 Functionalization of GQDs with mPRα

GQDs were manually prepared from citric acids. The preparation of GQDs was confirmed by visible fluorescence under UV light and by fluorescence scanning. GQDs solution showed blue color when exited by violet light bulb (Fig. 1A). The fluorescent peak was observed at 460 nm by excitation at 360 nm (Fig. 1B). Recombinant hmPRα was purified by two steps of column chromatography, Ni-NTA column and Cellufine Amino column chromatography, which was previously determined to be an effective resin for the purification of mPRα protein (Fig. 1C) [34]. Then, we tried to couple prepared GQDs with purified mPRα proteins. By a standard EDC/NHS reaction, GQDs were coupled with mPRα proteins on their amino groups through peptide bonds (Fig. 1C). Formation of peptide bonds between GQDs and mPRα proteins was confirmed by FTIR analysis (Fig. 1D). The peaks shown in Figure 1D at 1400 and 1593cm^{-1} indicate the presence of C=C stretching and carboxyl (C=O) group, respectively. A board peak at 3400cm^{-1} confirms the characteristic of hydroxy (-OH) group from carboxyl group. After surface functionalization of mPRα, a strong attached peak at 1644cm^{1} that assigned as the amide group appeared with the increased intensity at 1593cm^{-1}. In addition, GQD-mPRα showed another peak at 1710cm^{-1} which correspond to the C-N stretching. These specific peaks strongly suggested that prepared GQDs successfully coupled with mPRα. By TEM observation, average size of GQD-mPRα particle was determined as 9.5 nm (Fig. 1E).

178 Then, we analyzed the spectrometric characteristics of GQD-mPRα prepared in this
179 study. The maximum fluorescence intensity of the prepared GQD-mPRα was observed at an
180 excitation wavelength of 370 nm, and the peak fluorescence from GQD-mPRα was observed
181 at 470 nm (Fig. 2A). The excitation wavelength was slightly shifted to a longer wavelength of
182 360 nm for free GQDs.

183 *3.2 Specific binding of progesterone in P4-BSA-FITC with GQD-mPRα.*

184 Then, we tried to detect the binding of GQD-mPRα and P4-BSA-FITC. We set the
185 excitation wavelength for the reaction mixture to 370 nm, which was the maximum excitation
186 wavelength of GQD-mPRα. Scanning of the GQD-mPRα and P4-BSA-FITC reaction
187 mixture showed twin peaks when excited at 370 nm (Fig. 2B). The first peak at 470 nm
188 corresponded to free GQD-mPRα. The second peak at 520 nm corresponded to the mixed
189 fluorescent peak of GQD and FITC. The second peak of fluorescence at 520 nm was reduced
190 by the addition of free progesterone (Fig. 2B). The results suggested that a part of this peak
191 was from FITC-labeled progesterone that bound with GQD-mPRα by exciting FITC by
192 fluorescence from GQDs with a FRET mechanism. It is highly possible that the reduction in
193 fluorescence was caused by competitive binding of free progesterone to the steroid binding
194 site of mPRα and release of P4-BSA-FITC (Fig. 3). According the results, we measured the
195 excitation wavelength as 370 nm and the emission wavelength as 520 nm for the binding
196 assay. Under this condition, GQD-mPRα showed a reduction in fluorescence intensity at 520
197 nm upon the addition of the mPRα agonist progesterone. In contrast, GQD-BSA showed no
198 change in fluorescence intensity at 520 nm (Fig. 2C and 2D). The specificity of this assay
199 was further confirmed by assays of various steroids and compounds related to progesterone
200 (Fig. 2E). Only progesterone and 17α-hydroxyprogesterone showed competitive binding
201 activity against P4-BSA-FITC. In contrast, other steroids, estradiol, testosterone,

corticosterone and cortisol, showed no activity even at high concentrations. Thus, we concluded that we succeeded in detecting the binding of mPRα and its agonist progesterone. A model for the mechanism of changing fluorescent intensity by changing of specific binding of P4 in P4-BSA-FITC and steroid binding site in GQD-mPRα (Fig. 3). The decrease of fluorescent that caused by FRET between GQD and FITC induced by binding of free P4.

4. Discussion

Previously, we succeeded in purifying a relatively large amount of human mPRα protein with hormonal binding activity [31]. By using purified recombinant proteins, we established a way to detect the specific binding of steroids to the mPRα protein, which is expected to enable high-throughput screening of mPRα agonists or antagonists. The selectivity of steroids specific for progestins was confirmed. Among the four kinds of steroids, only progesterone and its analogs showed binding. In contrast, estradiol-17β, testosterone and cortisol did not show any binding activity, as reported by the results of a binding assay using ^{3}H-labeled steroid and recombinant proteins expressed in cancer cells [6]. Antagonists against nuclear progesterone receptor (PR), mifepristone (RU486), did not show competitive binding activity against P4-BSA-FITC. This result was consistent with reported results that showed that mifepristone did not bind to mPRα [6]. These results suggested that our established binding assay can detect the specific binding of progesterone to its steroid binding site.

We have already established and reported several assays for agonists or antagonists of mPRα [35]. Initially, we reported a steroid binding assay using ^{3}H-labeled steroids and cell membranes containing recombinant mPRα [36]. Although the assay gave us confident

results, manual operation using cell culture and test tubes is laborious and assaying large numbers of samples is difficult. Classical techniques and *in vitro* oocyte maturation assays using oocytes are still useful for analyzing mPR-interacting chemicals [37,38]. However, this technique requires fully developed oocytes that are ready to mature, which means that female animals must be ready to spawn. Thus, the method requires a large number of animals kept under appropriate conditions, and it is almost impossible to perform a large number of assays. We also found *in vivo* induction of oocyte maturation and ovulation by simply adding chemicals into the water in zebrafish [39]. Although this method enables the assay of the effect of chemicals on mPRs *in vivo*, the number of fish required for the assay is extremely high. Thus, the application of *in vivo* assays is limited for several selected compounds. Most recently, we established a cell-based chemiluminescent assay using a functionally modified luciferase gene [40]. The assay enables the detection of the function of agonists that induce changes in intracellular cAMP levels. However, the assay demands high costs, and it is difficult to obtain stable data due to difficulties in preparing cells under constant conditions. The established assay in this study using purified recombinant protein enables large numbers of assays with the same batch of purified fractions. Even though it can be improved on a smaller scale, more than 10 96-well plate assays are enabled by using the same preparation of GQD-mPRα. Thus, a highly reproducible assay for 1000 tests is enabled even on a laboratory scale. The changes in fluorescent intensity was small for this assay. However, high reproductivity recovered this disadvantage and produced significant differences. It can be speculated that a small magnitude of change reflects the exchange of progesterone on a single steroid binding site on mPRα.

In this study, we established a binding assay for human mPRα that enables the high-throughput screening of compounds that act on mPRα. This assay will lead to the identification of new compounds for pharmaceuticals.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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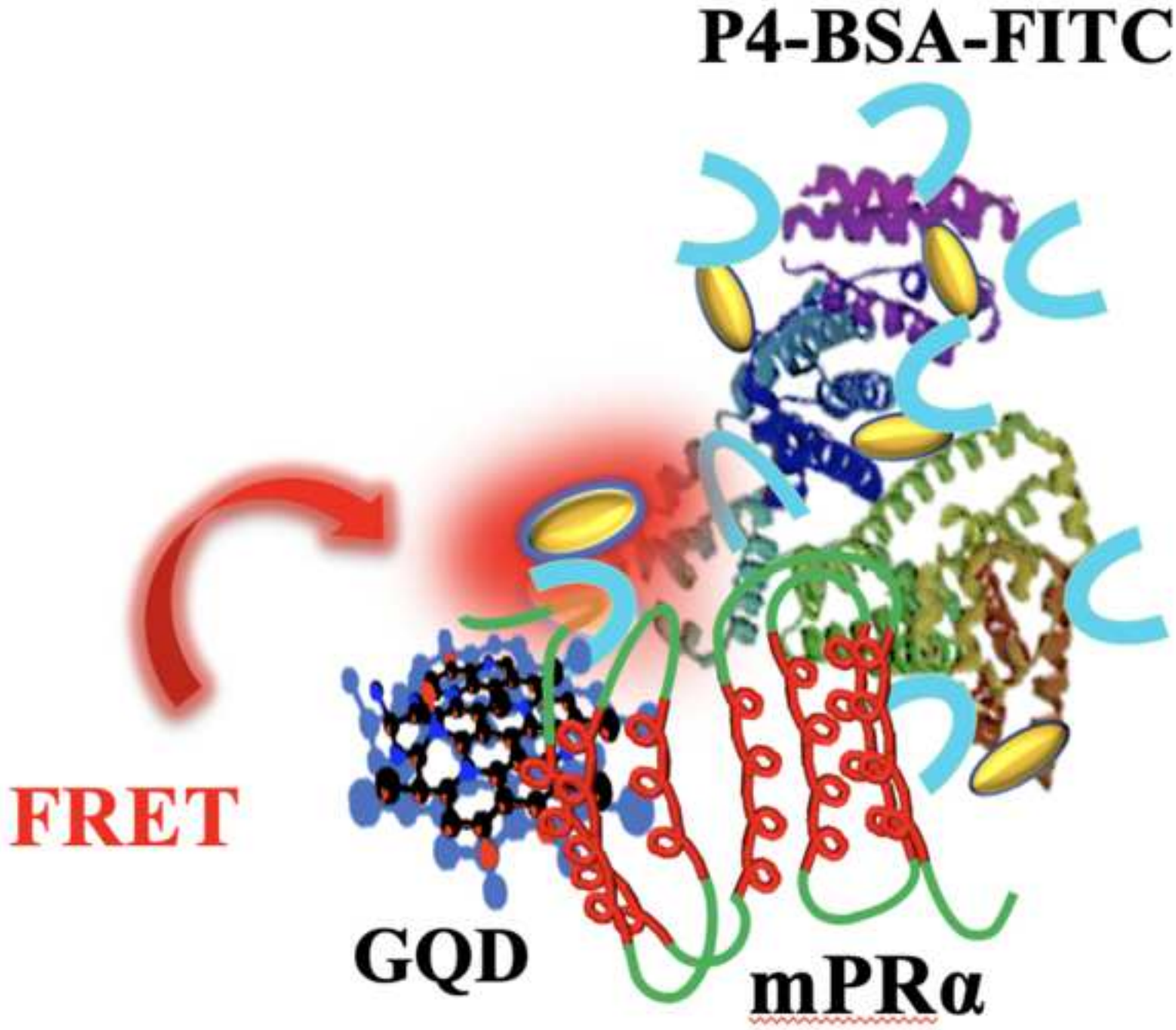
Figure legends

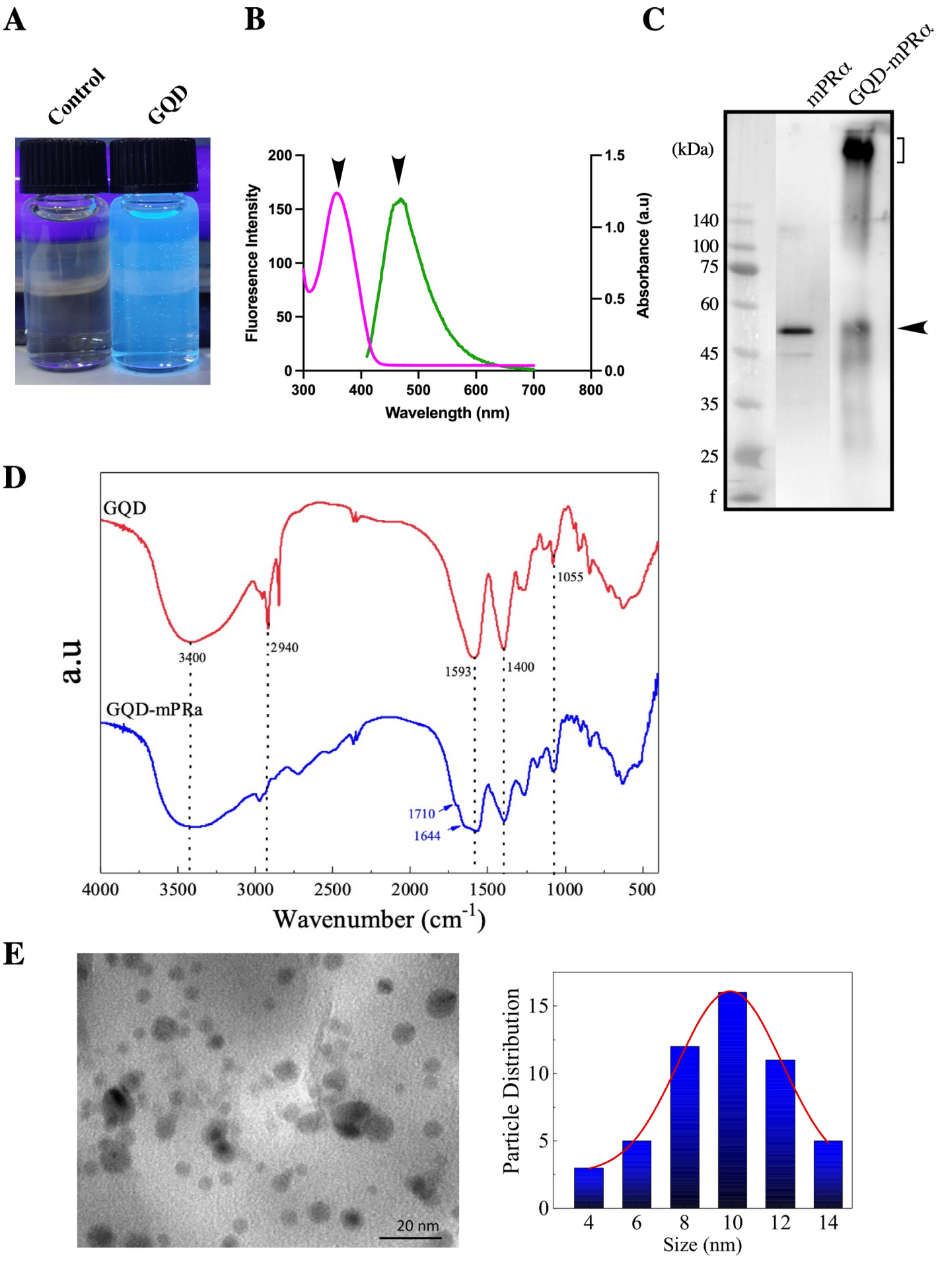
Fig. 1. Preparation of GQD, human mPRα protein and GQD-mPRα. (A) Photograph of prepared GQD solutions. A glass bottle with GQDs solution showed blue color (right panel) comparing with glass bottle with water (left panel) when exited by violet light bulb. (B)

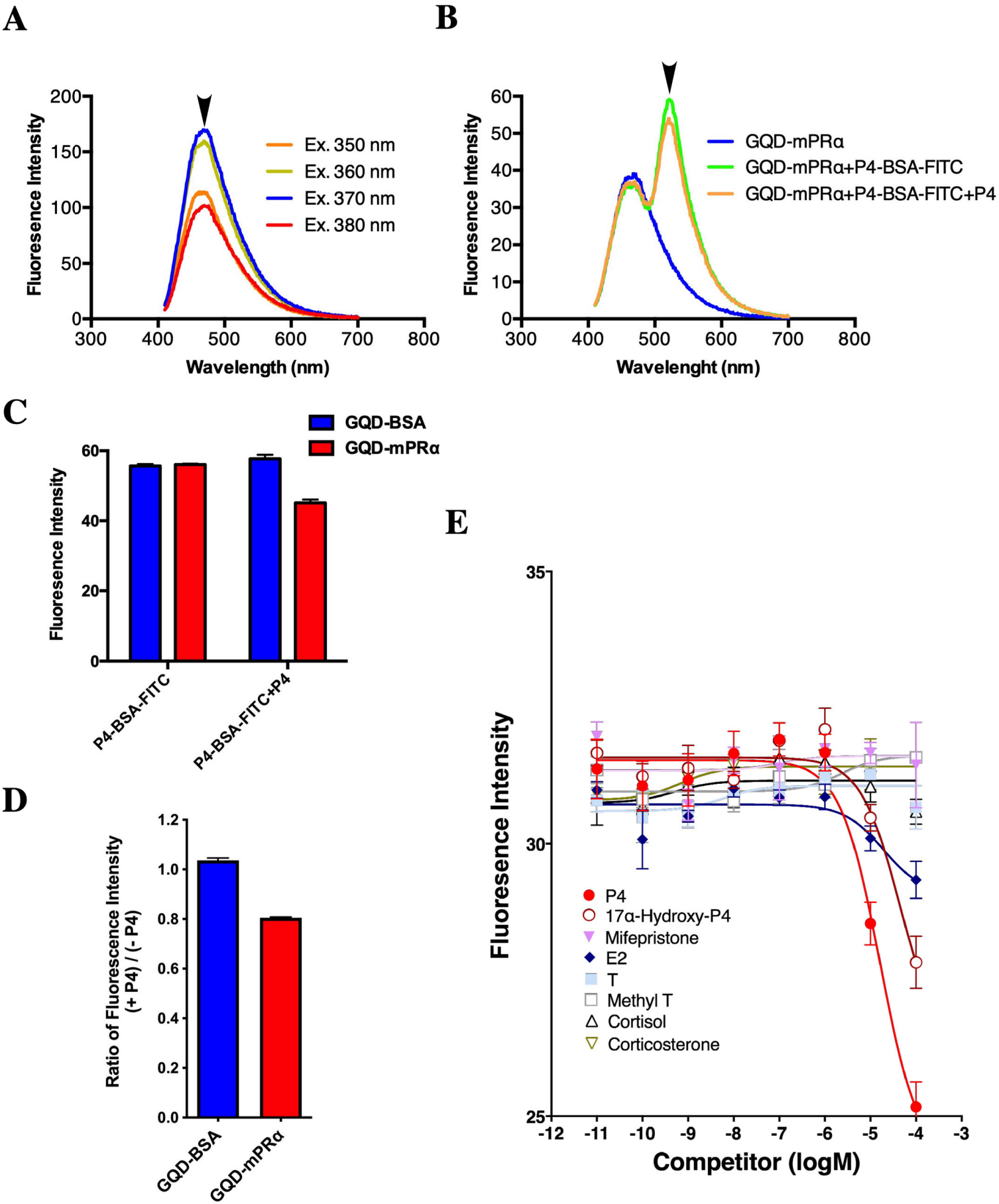
Absorbance scanning pattern (magenta) and fluorescent scanning pattern with excitation at 360 nm (green) of GQD. The peak of absorbance at 360 nm and fluorescence at 460 nm is indicated by arrows. (C) Western blot analysis of mPRα and GQD-mPRα. The protein band of mPRα is indicated by an arrowhead. The bands of GQD-mPRα are indicated by a bracket. (D) FTIR scanning data of GQD (red line) and GQD-mPRα (blue line) solution. (E) TEM observation of GQD-mPRα. Sizes of observed GQD-mPRα particles were determined and their distribution was summarized.

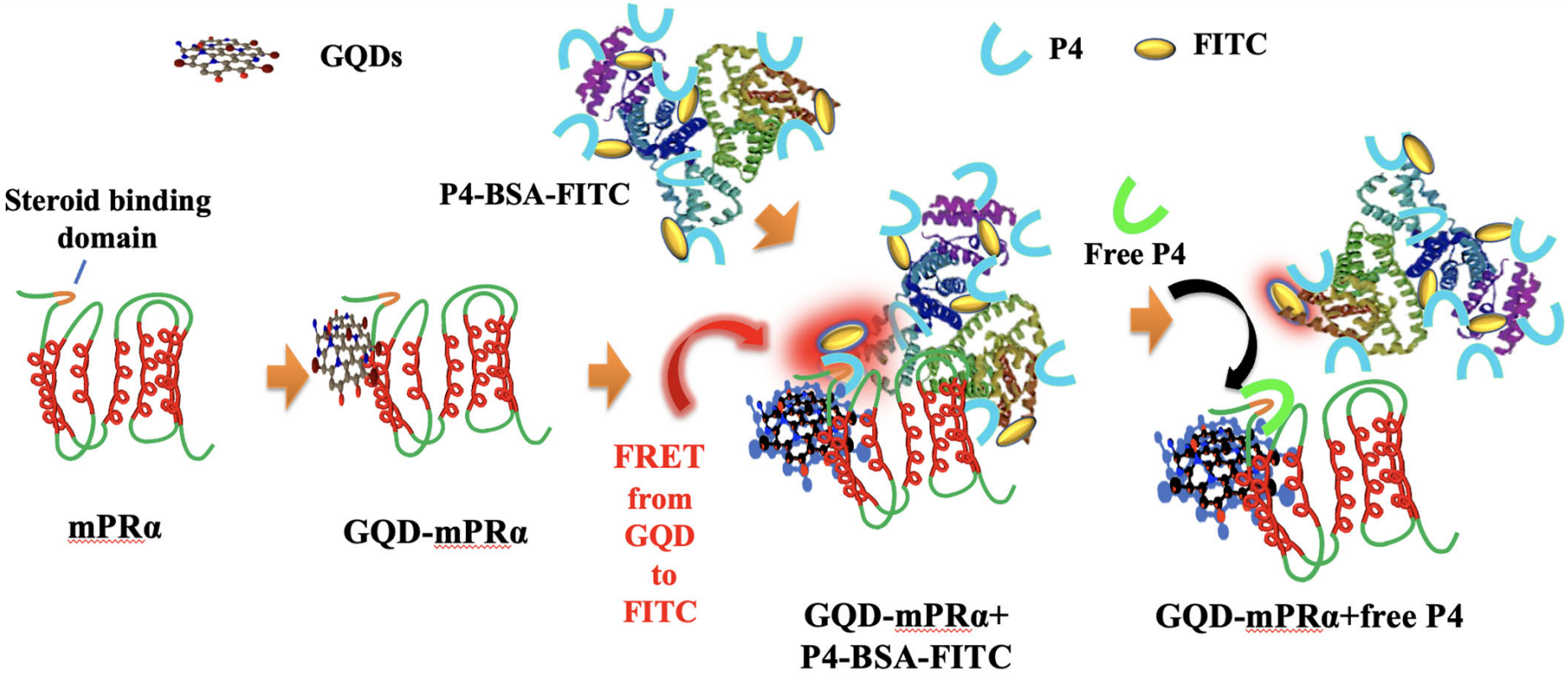
Fig. 2. Fluorescence characteristics of GQD-mPRα and detection of specific interactions with P4-BSA-FITC. (A) Fluorescent scanning pattern of GQD-mPRα with different excitation wavelengths. The peak of fluorescence at 470 nm is indicated by the arrow. (B) Fluorescent scanning pattern of the reaction mixture with or without free P4. The peak of fluorescence at 520 nm is indicated by the arrow. The fluorescence scanning pattern of free GQD-mPRα is also indicated. (C) Measurement of the fluorescence of the reaction mixture under established conditions (Ex 370 nm and Em 520 nm). Fluorescence from GQD-BSA (blue bar) and GQD-mPRα (red bar) mixed with P4-BSA-FITC are compared (P4-BSA-FITC). The effects of the addition of free P4 into the reaction mixture are also indicated (P4-BSA-FITC+P4). (D) The ratios of fluorescence in the absence and presence of free P4 for competitive antagonists are indicated. (E) Competition of the binding of P4-BSA-FITC with GQD-mPRα by steroids and their analogs. The dose-dependent effects of steroids (progesterone (P4), 17α-hydroxyprogesterone, estradiol-17β (E2), testosterone (T), cortisol, corticosterone) and their analogs (mifepristone (RU486), methyl-testosterone (Methyl T)) were assayed by the established assay.

Fig. 3. Schematic diagram of GQD-mPRα and predicted mechanisms of interaction between P4-BSA-FITC. Expected interaction between GQD-mPRα and P4-BSA-FITC (GQD-mPRα+P4-BSA-FITC) is indicated. It is expected that red fluorescence from FITC caused by fluorescence from closed GQDs by the binding of P4-BSA-FITC. The interaction between GQD-mPRα and P4-BSA-FITC is released by free P4, and fluorescence from FITC decreases. Diagrams of each compound (GQD, mPRα, GQD-mPRα, progesterone (P4), FITC are indicated.


P4-BSA-FITC
FRET
GQD
mPRα


A
Control
GQD
B
Fluoresence Intensity
Absorbance (a.u)
Wavelength (nm)
C
mPRα
GQD-mPRα
(kDa)
140
100
75
60
45
35
25
f
D
GQD
GQD-mPRa
a.u
3400
2940
1593
1400
1055
1710
1644
Wavenumber (cm-1)
E
20 nm
Particle Distribution
Size (nm)


A
Fluoresence Intensity
Wavelength (nm)
Ex. 350 nm
Ex. 360 nm
Ex. 370 nm
Ex. 380 nm
B
Fluoresence Intensity
Wavelenght (nm)
GQD-mPRα
GQD-mPRα+P4-BSA-FITC
GQD-mPRα+P4-BSA-FITC+P4
C
GQD-BSA
GQD-mPRα
Fluoresence Intensity
P4-BSA-FITC
P4-BSA-FITC+P4
D
Ratio of Fluorescence Intensity (+ P4) / (- P4)
GQD-BSA
GQD-mPRα
E
Fluoresence Intensity
Competitor (logM)
P4
17α-Hydroxy-P4
Mifepristone
E2
T
Methyl T
Cortisol
Corticosterone


GQDs
P4
FITC
P4-BSA-FITC
Steroid binding domain
Free P4
mPRα
GQD-mPRα
FRET from GQD to FITC
GQD-mPRα+ P4-BSA-FITC
GQD-mPRα+free P4