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Bioconjugation of the estrogen receptor hER$_\alpha$ to a quantum dot dye for a controlled immobilization on a SiO_2 surface

C. Cherkouk *, L. Rebohle, W. Skorupa

Institute of Ion Beam Physics and Materials Research, Forschungszentrum Dresden Rossendorf, POB 510119, D-01314, Germany

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ABSTRACT

We investigated the immobilization of the estrogen receptor hER$_\alpha$ on silanized SiO_2 surfaces for biosensor applications. The conjugation of the estrogen receptor hER$_\alpha$ to the quantum dot dye QD655 was achieved. In order to obtain an optimal immobilization of the estrogen receptor hER$_\alpha$ on the functionalized SiO_2 surface, the bioconjugate hER$_\alpha$-QD655 (Rcpt-qd655) solution was prepared with a higher molar ratio of 10–15 between the QDs and the receptors. A blue laser with an excitation wavelength of 405 nm was used for photoluminescence spectroscopy (PL) investigations to monitor the bioconjugate Rcpt-qd655 immobilization on the silanized SiO_2 surfaces with three different functional groups, namely NH_2, $-COO^-$, and –SH. Several wash processes were applied to remove the excess receptors from the surface after the immobilization. A Fourier transform infrared spectroscopy (FTIR) was used to control the biofilm background after each wash of the receptor-coated surface which allows the optimization of the immobilization protocol. In order to test its stability a quartz crystal microbalance (QCM) was employed and the receptor density was calculated. Finally the optimal biolayer (silane film + hER$_\alpha$ receptor) was tested for measurements of 17ß-estradiol (E2) with a concentration of 1 μM in waterish solution. The measurement concept outlined in [L. Rebohle et al., Vacuum 83 (2009) 24–28] was applied. The whole system was investigated by PL, which exhibits two color signals, namely from the receptor and the detected E2 molecules.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are environmental pollutants that interfere with human hormones and influence the self-hormonal regulation of the human population and wildlife [1,2]. The contamination of the raw water sources comes from human sewage, e.g., oral contraceptive (ethinylestradiol EE2), or animal food and leads to several effects, e.g., gentoxic, neurobiological, and carcinogenic [3,4]. A common endocrine disruptor includes the environmental estrogen. For this reason, there is an increasing demand for fast detection methods for estrogenic activity in water, but current detection methods are based on laboratory facilities [5]. Further miniaturization can be achieved by using light sources integrated on a chip. In this concept, known as direct fluorescence analysis and outlined in [6,7], a metal–oxide-semiconductor-based light emitting device (MOSLED) is placed directly beneath the bioactive layer consisting of a silane coupling agent, a receptor, and a dye-labeled analyte. The surface of the Si-based light emitter can be functionalized by applying the SSC (spraying spin coating) method which avoids processes that are harmful to the MOSLED and is published in [8].

The controlled receptor immobilization on a silanized sensor surface plays a significant a role for an efficient and specific binding of the estrogen molecules to the so-called binding pocket of the receptor [9].

As shown in Fig. 1a the immobilization of the hER$_\alpha$ receptor on the functionalized MOSLED surface should be achieved in such a way that the receptor is well oriented toward the top of the biofilm of the MOSLED. The simulation (code 1HCQ from the protein data bank given in [10]) sketched in Fig. 1a shows this interaction of the hER$_\alpha$ receptor with 17ß-estradiol (E2) which exhibits more than three specifically bound E2 molecules per receptor. The experimental techniques for systematically controlling the receptor immobilization are limited. Through ellipsometrical investigations the thickness of the targeting molecule can be determined [11], and a suitable theoretical model for analyzing the results is necessary. Other methods like QCM (quartz crystal microbalance) are able to detect the adsorbed mass of the biomolecule by a change in the frequency of the gold membrane [12], which will be applied in this work to prove the immobilized receptor protocol. In order to increase the specificity of the biolayer the receptors were modified to build hER$_\alpha$-QDs655 (Rcpt-qd655) bioconjugates. The molecular shell of the nanoparticle is well defined after the conjugation.

* Corresponding author.
E-mail address: c.cherkouk@fzd.de (C. Cherkouk).

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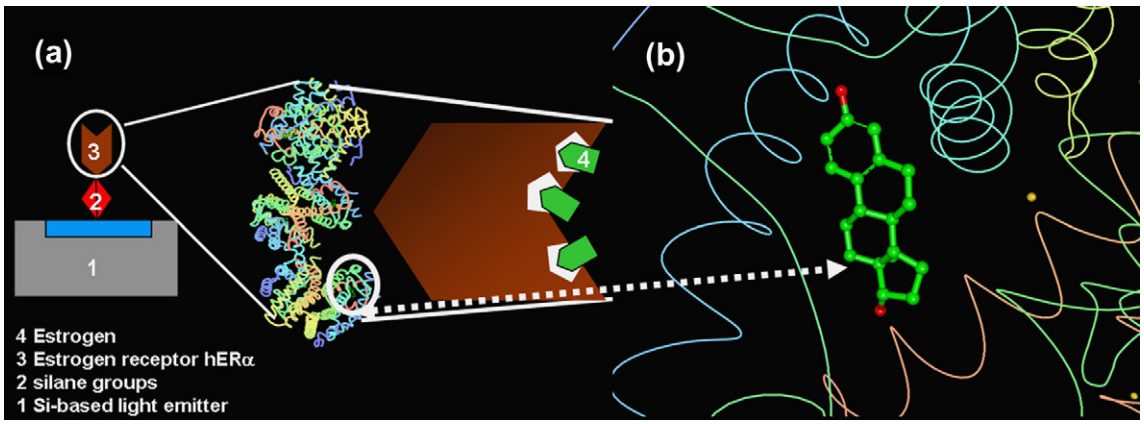

Fig. 1. Left part: Schematic illustration of the adjustment of the biocomponent on the top of the Si-based light emitter (a.1) after the silanization with silane groups (a.2) and the immobilization of the hER_α receptor (a.3). The interaction between the human receptor hER_α and the 17ß-estradiol (E2) was simulated by using the code 1HCQ from protein databank given in [10]. Right part is a sketch of a zoomed simulation part of the E2 docking into the receptor.

The immobilization of the estrogen receptor on the silanized SiO_2 surface occurred under a certain angle which makes the binding pocket of the receptor accessible for estrogen docking. Finally the whole sensor concept was applied on a waterish sample with an estrogen concentration of 1 µM to test the sensitivity of the system. The dye-labeled E2 solution was realized by using a second QDs800 dye with an emission wavelength at 800 nm to build E2-QDs800 (E2-qd800). The PL signal at 800 nm is proportional to the concentration of the E2 molecules in the waterish solution which expresses the affinity between the E2 and the hER_α receptor.

In this study the immobilization of the conjugated estrogen receptor hER_α with QDs655 dye on the silanized SiO_2 surfaces was investigated. Fourier transform infrared spectroscopy (FTIR) was used to optimize the wash procedure. PL spectroscopy was applied in order to control the immobilization to different silane layers by monitoring the 655-nm wavelength. The immobilization of the estrogen receptor was optimized to obtain a better analyte-receptor-coated surface for E2 concentration measurements at 800 nm.

2. Materials and methods

2.1. Materials

SiO_2 substrates were 200-nm-thick SiO_2 layers deposited by plasma-enhanced chemical vapor deposition (PECVD) on top of a 4-in. {1 0 0} oriented n-type silicon wafer. (3-Aminopropyl)-trimethoxysilane (APMS) (purity >97%), (3-mercaptopropyl)-trimethoxysilane (MPMS), a 0.27% (w/v) buffer solution of 2-(*N*-morpholino)ethanesulfone acid hydrate (MES) ($C6H13NO3S{\cdot}xH_2O$) in ultrapure water (18 MΩ), and a phosphate-buffered saline (PBS) solution were purchased from Sigma Aldrich Germany and used without further purification. The amount of 750 pmol of the recombinant human estrogen receptor hER_α (65 kDa), which is produced from baculovirus-infected insect cells and purchased from Sigma Aldrich Germany, was supplied as purified protein in 50 mM Tris–HCl, pH 8.0, 500 mM KCl, 2 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, and 10% glycerol. The 1 µM QDs-carboxyl conjugates (QDs 655 and 800 consist of CdSe core and ZnS shell with emission wavelengths at 655 and 800 nm, respectively) were purchased from Invitrogen Europe. For the bioconjugation of the receptor to QDs dye a solution of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) (40 mM, purity 97%), *N*-hydroxy-succinimide (NHS) (63 mM, purity 98%), 2-aminoethanol, and spin centrifuge tube filters with nylon membrane of 0.22 µm pore size were used (Sigma Aldrich Germany). Two steroid hormones of 17ß-estradiol and 17ß-estradiol 17-humisuccinate were prepared for the test measurements and for the bioconjugation of the E2-qd800, respectively (Sigma Aldrich Germany). For QCM measurement the quartz crystals were purchased from L.O.T. Oriel Germany.

2.2. Methods

2.2.1. SSC (spraying spin coating)

Prior to silanization the surface of the SiO_2 substrates was cleaned by immersion in an isopropanol solution for 10 min followed by sonication and immersion in methanol and acetone for 10 min each.

After the hybridization with MES Catalyse solution to obtain active hydroxyl groups on the SiO_2 surface, the silanization is achieved by spraying the silane solution to react directly with silanol groups on the surface. Further details of the SSC method are described in [8].

2.2.2. Immobilization of the estrogen receptor hER_α on the silanized SiO_2 surface

The bioconjugate hER_α-QD655 (Rcpt-qd655) solution was prepared with a higher molar ratio of 10–20 between the QDs and the hER_α receptors and filtrated by using the spin centrifuge tube filters. The volume amount of the used QDs dye was calculated with the formula [13]

$$\text{dye}\ (\mu\text{l}) = \text{Receptor}\ (\text{mg/ml}) \cdot 0.05\ \text{ml} \cdot f \cdot \text{MR} \cdot \text{MW(QDs-dye)/MWReceptor)},$$

where MR is the molar ratio between the QDs dye and the receptor hER_α in the Rcpt-qd655 solution, f is unit conversion factor ($f = 100$), MW(QDs dye) = 30,000 g/mol, MW(receptor) = 65 kDa are the molar weight of the QDs dye and the receptor, respectively. The bioconjugation protocol modified the QDs containing carboxyl groups in a two-step process to form an amide bond as shown in Fig. 2 (steps 1–2) and is described in [14].

The silanized substrates were inserted in special cylindrical tubes and placed in a glass chamber, where 20 µl of the Rcpt-qd655 conjugates was pipetted for each sample. The immobilization chamber was coupled to a shaker to obtain a spherical movement of about 350 rpm. The samples were incubated for 1 h. Finally the substrates were washed with phosphate-buffered saline three times and dried by using nitrogen streaming.

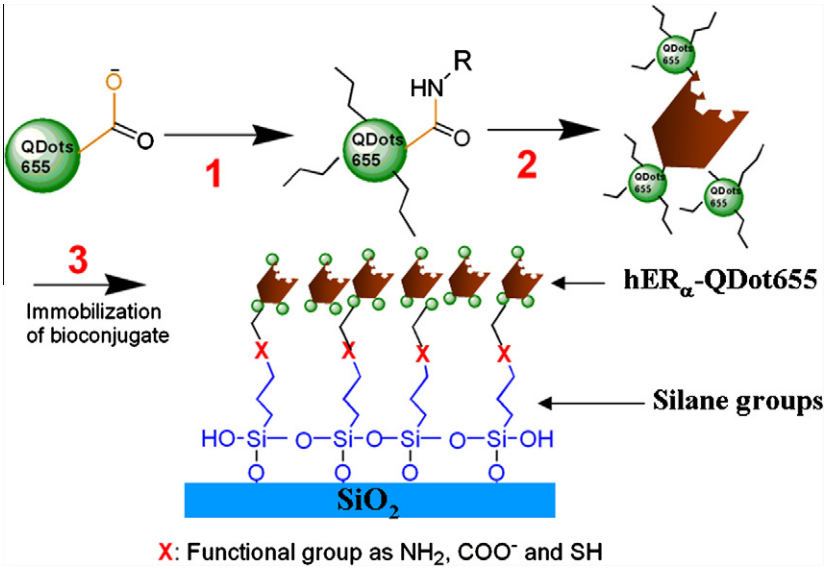

Fig. 2. The bioconjugation protocol of the estrogen receptor hER$_\alpha$ to quantum dot (steps 1–2) and the immobilized Rcpt-qd655 on the silanized SiO_2 surface (3) and described in [14].

2.2.3. Detection of E2 in waterish solution

In order to test the capture of the QDs-labeled receptor Rcpt-qd655 on the silanized SiO_2 surfaces, E2 was selected as the steroid with the highest affinity to the receptor hER$_\alpha$ [9]. The bioconjugate solution E2-qd800 was prepared by using the same protocol as in the case of the bioconjugation of the Rcpt-qd655 solution. The QD800 will be bound in the 17th position of the E2 molecules because of the high reactivity of the NHS derivate to the amide bound to the modified QD800. After the immobilization of the bioconjugate Rcpt-qd655, the biofilm was passivated by washing with ethanolamine to deactivate the moiety of the reactive silane groups on the surface. This step should increase the specificity of the binding between E2 and the receptor. The test measurement protocol with E2 in the waterish solutions is outlined in [6]. After an incubation time of 30 min the samples were washed with PBS and dried by using nitrogen streaming.

The experimental setup for the photoluminescence spectroscopy measurements consists of an external 405 nm laser and a microscope which is connected with a CCD camera for light detection (range between 350 and 900 nm).

2.2.4. FTIR spectroscopy

A Magna FTIR Fourier transform infrared spectrometer (Nicolet) with an incident angle of 9° was used to check the film composition after silanization and the adsorption with lysine molecules as a function of the pH values. Spectral analysis was performed in the region of [4000, 400] cm^{-1} and [2000, 400] cm^{-1} for the silanized and adsorbed surface, respectively.

2.2.5. X-ray photoelectron spectroscopy

The chemical composition of the unmodified QDs and with E2 coverage was analyzed by X-ray photoelectron spectroscopy (XPS) with the help of a Microlab 310 F device (Fisions Instruments). The XPS measurements were carried out at a base pressure of about 5×10^{-10} mbar using an Mg Kα X-ray (1253.6 eV) source. The electron analyzer pass energy in the XPS high-resolution scans was constantly 10 eV. The incidence angle of the X-ray beam amounts to 60°, and the line of detection is parallel to the sample's normal.

2.2.6. Quartz crystal microbalance

In order to characterize the affinity between surface-immobilized receptor hER$_\alpha$ and soluble E2 molecules in solution, an E4 Qsense device from Qsense for the quartz crystal microbalance QCM-D measurements in flow mode was used. Prior to QCM measurement the quartz crystals with a gold film and an additional 50-nm-thick SiO_2 layer were cleaned. The receptor hER$_\alpha$ was immobilized by means of a Nano-Plotter from Gesim Gmbh in Germany, which allows the making of array structures. For the nanoplotting a volume of 25 µl of the receptor hER$_\alpha$ in PBS buffer was used.

3. Results

3.1. FTIR spectroscopy

The wash processes were applied to remove the excess receptor from the surface and thus to increase the specific binding.

Fig. 3a shows a FTIR spectrum of the silanized surface with APMS. The amino group at 1600 cm^{-1} is one of the three types of

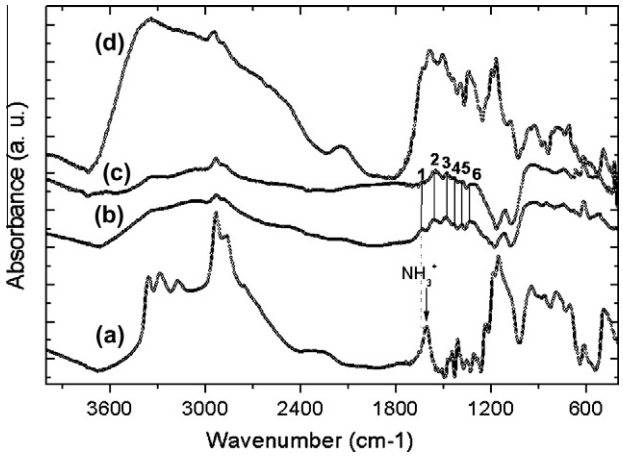

Fig. 3. FTIR spectra of the following samples: SiO_2 silanized with SSC-APMS before (a) and after immobilization: with filtering of the Rcpt-qd655 solution and wash step using the buffer solution PBS (b) without filtering of the Rcpt-qd655 solution and wash step with PBS (c) and without any wash treatments (d).

the functional groups together with thiol and carboxyl group at 2500 and 1600 cm^{-1}, respectively, which were realized by applying the SSC method. As shown in Fig. 3b after the immobilization of the filtered Rcpt-qd655 the biofilm causes an underground about 3355 cm^1 which is smaller compared to Fig. 3d without the wash. The FTIR absorbance peaks between 1635 and 1180 cm^1 are assigned to the molecular shell of the QDs. These fingerprints cannot be found in Fig. 3a of silane film before the immobilization. The quality of the biofilm in Fig. 3c shows that the wash treatment is enough to remove the excess Rcpt-qd655 from the surface. Especially the positions of the absorbance peaks 1–6 (i.e., 1640, 1558, 1480, 1427, 1384, and 1338 cm^1) between the spectra in Fig. 3c and b appear at the same positions, indicating a comparable binding behavior of the molecular shell of Rcpt-qd655 to the amino groups of the silane film. This result will be confirmed by PL measurements. Therefore the wash procedure with PBS, but without a prior filtration, will be applied in the further biofilm fabrication.

3.2. XPS spectroscopy

Fig. 4a shows the XPS spectra overview spectrum of the unmodified QDs. All compounds of the QDs were measured except selenium. However, in the case of the unmodified QDs, three binding energies were measured for Cd at 403.2, 408.2, and 414.5 eV for Cd3d, $Cd3d_{5/2}$, and $Cd3d_{3/2}$, respectively [15]. Only one peak from the shell of the QDs for the S2p energy level was measured at 157.3 eV [16]. All these bonding energies disappear by the covered QDs with E2 except the Zn peak.

Fig. 4b shows XPS spectra of the binding energy of $Zn_{1/2,3/2}$ from the unmodified QDs and with E2 coverage, respectively. Three binding energies were measured for the unmodified QDs (open circles) which is centered at 1016.6 eV and consists of the two satellites at 1011 and 1020 eV [17], which cannot be observed in the spectrum in Fig. 4b (dotted line) of the E2-coated QDs with E2. However, the binding energy of the level $Zn_{1/2,3/2}$ was measured at 1015.8 eV and shifted to lower binding energy compared the unmodified QDs. Obviously, the XPS investigations described qualitatively of the bioconjugated QDs. The binding sites are still unknown between the receptor QDs, on one hand, and the QDs-silanized surface, on the other hand, which could be due to the depth limitation of XPS.

3.3. PL spectroscopy

3.3.1. Immobilization of the Rcpt-qd655

Fig. 5 shows the PL spectra of the immobilized Rcpt-qd655 on three silanized SiO_2 surfaces with amino, carboxyl, and thiol groups. The PL signal was observed by the three silanized surfaces; i.e., no loss of the fluorescence intensity was observed regarding the particle stability which is influenced by the choice of the buffer, the clumping, or the precipitation of the QDs. Ostuni et al. proved the inertness, i.e., an adsorbing, of the fibrinogen protein and the lysozyme enzyme on the surfaces covered with self-assembled monolayers which consist of a simple amide and of amides based on amino acids [18].

This fact suggests that the modified QDs with amide groups are bound to the receptor hER_α. The molar ratio of the Rcpt-qd655 bioconjugates must still be controlled in such way that the receptor will be linked through the QDs shell on the silanized SiO_2 surfaces (Fig. 2). As shown in Fig. 5a the highest PL intensity was measured by the silanized surface with carboxyl groups. This result can be explained by a coulomb interaction between the COO^- groups originating from the silane film and the amide bond to the modified receptor. The PL quantum yields of the immobilized Rcpt-qd655 on the thiol and amino groups in Fig. 5b and c, respectively, are relatively the same. The peak maxima of their signals are blue-shifted to shorter wavelengths of 652 nm (amino groups) and 647 nm (thiol groups) compared to that of the immobilized Rcpt-qd655 bound to the carboxyl groups. This can be explained by changing the confinement energies inside the QDs compounds [19]. This result confirms the chemical shift observed by XPS measurements for the unmodified and covered QDs.

Fig. 6 shows the PL spectra of the immobilized Rcpt-qd655 bioconjugates on the silanized SiO_2 with carboxyl groups by using tree molar ratios between the hER_α and the QD655, namely MR = 13 (Fig. 6a), 6 (Fig. 6b), and 3 (Fig. 6c). The PL signal originating from the bioconjugate Rcpt-qd655 disappears at MR = 3, which will be considered as a critical MR value. Further experiments for the detection of E2 in the waterish solution will be performed at the highest MR = 13 value.

3.3.2. The detection of E2 in waterish solution

In order to prove the adjustment of the different biocomponents on the silanized SiO_2 surface, the same concept as outlined in [6]

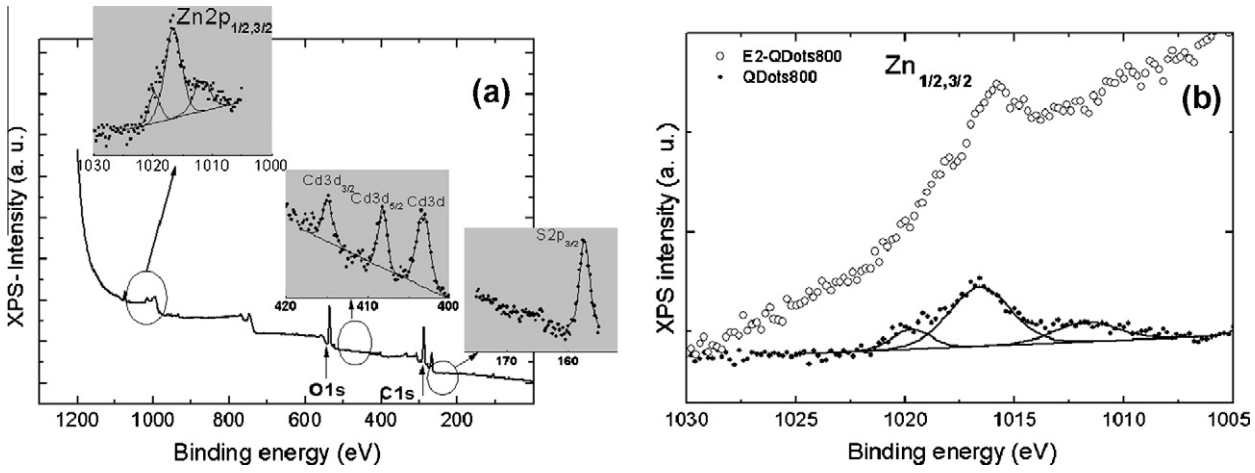

Fig. 4. (a) XPS survey spectra from the unmodified QDs dissolved in PBS to silanized SiO_2 surface with amino groups and the close-up surveys of $Zn_{1/2,3/2}$, Cd3d, $Cd3d_{5/2}$, $Cd3d_{3/2}$, and $S2p_{3/2}$, respectively. (b) XPS spectra of the energy level $Zn_{1/2,3/2}$ from the unmodified QDs 800 dissolved in PBS (open circles) and from E2-qd800 bioconjugates (dotted line) to silanized SiO_2 surface with amino groups.

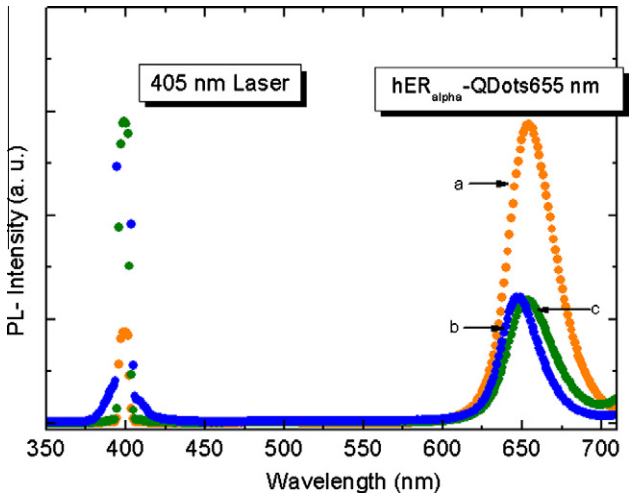

Fig. 5. PL spectra of immobilized Rcpt-qd655 bioconjugates on the silanized SiO_2 surface with carboxyl (a), thiol (b), and amino groups (c).

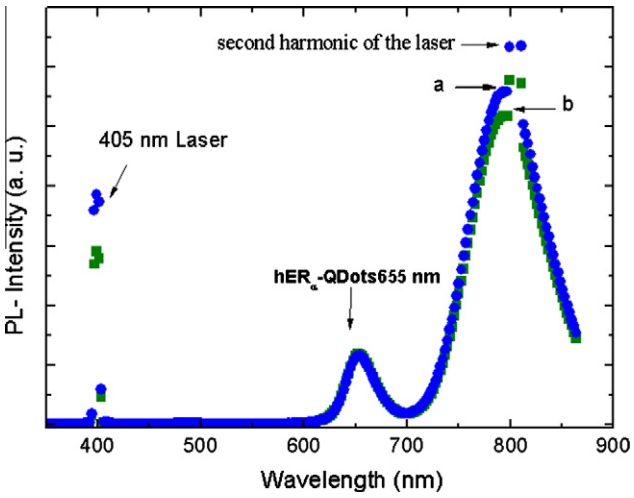

Fig. 7. PL spectra of Rcpt-qd655 bioconjugates immobilized on two carboxylated SiO_2 surfaces by using the detection concept outlined in [6] for measurements of two E2 concentrations of 1 mM and 1 μM in waterish samples. See experimental protocol for detailed conditions.

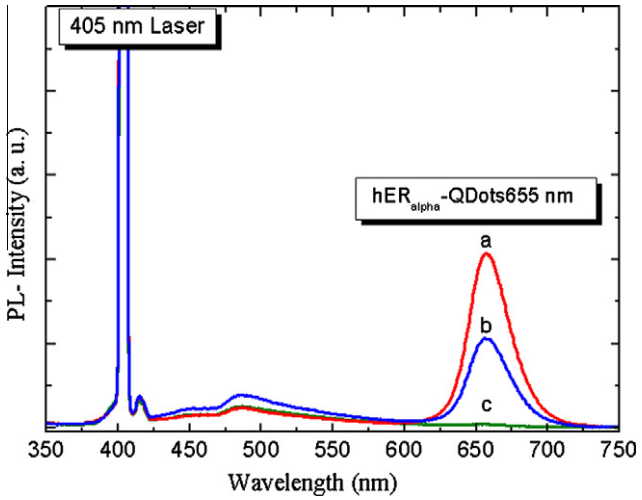

Fig. 6. PL spectra of immobilized Rcpt-qd655 bioconjugates on the silanized SiO_2 by using three molar ratio MR of Rcpt-qd655 for MR = 13 (a), MR = 6 (b), and MR = 3 (c).

was applied. A carboxyl-silanized SiO_2 surface was used to immobilize the Rcpt-qd655 bioconjugates. Two waterish samples with E2 concentrations of 1 μM and 1 mM were applied.

Fig. 7 shows the optical signal of the excitation laser sources at 405 nm, the signal of the immobilized Rcpt-qd655 at 655 nm, and the signal from E2-qd800. The second harmonic of the blue laser overlaps with the QD800 signal at 800 nm, but it remains distinguishable from each other. The signal of the immobilized Rcpt-qd655 appears with the same PL intensity for both water samples, which means that the immobilization of the bioconjugate of Rcpt-qd655 on the silanized SiO_2 surface is reproducible. However, the signal at 655 nm is smaller than the signal of the E2-qd800. This result can be related to the higher extinction coefficient of QD800 compared to the QD655 ($10^6\ M^{-1}\ cm^{-1}$) at the 405 nm excitation wavelength [20]. However, the signal of the E2-labeled QD800 is already saturated in the case of a 1 μM solution, for which reason this method can be easily applied for concentrations below the micromolar range. Actually, the needed detection limit of the estrogen in drinking water is about ng/l (∼ pM range).

3.4. QCM-D measurement

Furthermore, the sensor concept will be applied on the silicon-based light (MOSLED) emitter as integrated light source which is already published elsewhere [6,7]. The system will be integrated in a fluidic cell and the measurement of the estrogen concentration will be operated in a dynamic flow system. In order to quantify the immobilized hER$_\alpha$ by using such immobilization protocols, and especially the combination between a silane film as functionalization of the MOSLEDs surface and the estrogen receptor hER$_\alpha$, a QCM measurement was applied. This technique is based on quartz crystal oscillations in shear mode with a resonance frequency which is very sensitive to the mass and thus to the adsorbed molecules. The frequency difference will be monitored.

Fig. 8a shows the time evolution of the mass of E2 adsorbed on the biolayer (immobilized receptor hER$_\alpha$ + silanized SiO_2 surface). A slow equilibrium was reached after 12 min by a PBS wash. The

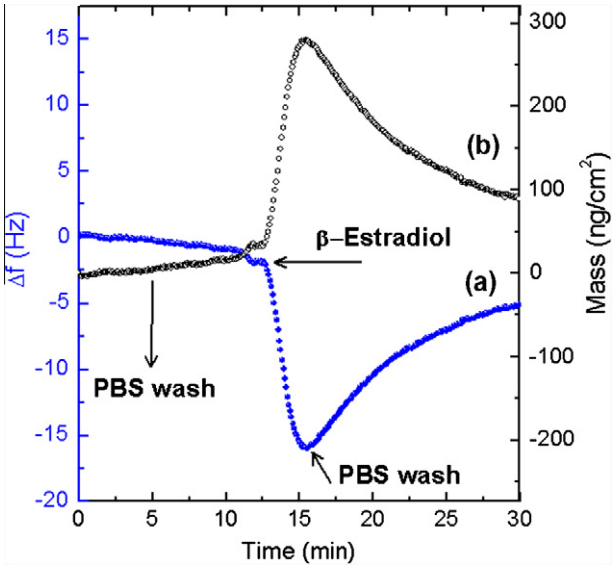

Fig. 8. QCM spectrum in flow mode to characterize the affinity between a surface-immobilized receptor hER$_\alpha$ and soluble E2 molecules (a) and the calculated Sauerbrey curve of the E2 adsorbed molecule density (b).

E2 waterish solution was injected. A rapid decrease of the frequency difference was observed which is related to an increase of the E2 adsorbed mass. Shortly after this step a second PBS wash was started, and within 15 min a slow increase of the frequency difference occurred; i.e., the adsorbed E2 molecules were partly removed. This could be related to the very short incubation time between the E2 flowing step and the second PBS wash step. However, Δf can be transferred into the mass change with an equation of the form [21]

$$\Delta f = -2f_0^2 \frac{1}{\sqrt{\mu_Q \rho_Q}} \frac{\Delta m}{A}$$

where Δf is the frequency difference, $f_0 = 850$ Hz is the resonance frequency of the whole system including the immobilized receptor hER_α + silane film, $\mu_Q = 4.4 \times 10^{11}$ (g/cm s^2), $\rho_Q = 2.65$ (g/cm^3), and A are the shear modulus, the density, and the surface area of the quartz crystal, respectively. As shown in Fig. 8b the maximum of the E2 adsorbed mass is about $\Gamma_{max} = 276$ ng/cm^2 which is equivalent to around 10^{14} E2 molecules/cm^2.

4. Discussion

Fig. 3 shows a smaller FTIR underground signal about 3355 cm^{-1} for washed samples with the immobilized bioconjugate Rcpt-qd655 on the silanized surface compared to the sample without a wash. This wash step is considered to be enough for rinsing unbound molecules from the surface and therefore increase the specific adsorption of the biocomponents on silanized the SiO_2 surface.

The immobilization of the bioconjugate Rcpt-qd655 on the three functionalized SiO_2 surfaces by means of the SSC method in Fig. 5 provided the highest PL signal in the case of the silane film with carboxyl groups, which proved the linking of the receptor to the surface via QD655. That means the reaction between the amide of the QDs (Fig. 2) and the carboxyl groups of the silanized surface was performed [18]. The molar ratio MR between the estrogen receptor hER_α and the QDs 655 dye was varied to realize the binding strategy schematized in Fig. 2. No PL signal could be seen at MR = 3.

The same PL signal of Rcpt-qd655 is shown in Fig. 6 which provides the same immobilized number of Rcpt-qd655 bioconjugates on the silanized surface at a constant MR value.

The PL peak in Fig. 7 caused by E2-qd800 is higher as expected for the 1 μM (Fig. 7a) E2 concentration compared to the PL signal for 1 mM (Fig. 7b) concentration. However the PL intensity difference of E2-qd800 (Fig. 7) is not big enough compared to the E2 concentration gap between 1 μM and 1 mM. This fact could be due to the contribution of the nonspecific adsorption of E2-qd800 on the surface. Thus, the wash process between the steps was not enough to decrease the nonspecific adsorption and therefore this process must be multiplied between the steps. However, it is likely that the detection system that we have described here will be integrated in a microfluidic system, which will so far achieve this condition. That means the detection system in dynamic flow can be optimized by rinsing with a buffer solution as long as the system is stabilized. This was demonstrated by applying the QCM technique in Fig. 8 to our biofilm. Finally the kinetic curve versus time of the adsorbed E2 was measured.

5. Summary

The conjugation of the estrogen receptor hER_α to the quantum dots dye (QD655) was achieved. We used three molar ratios MR = 13, 6, and 3 between the QDs and the receptor so that the immobilized bioconjugate Rcpt-qd655 was linked with a defined molecular shell, namely with amide of the QDs, on the silanized surfaces. This binding strategy allowed the controlling of the immobilized receptor hER_α and its orientation which is necessary for estrogen docking. PL measurements of the immobilized bioconjugate Rcpt-qd655 on three types of silanized SiO_2 surfaces revealed a higher PL intensity at 655 nm for the carboxylated SiO_2 surface. The E2-labeled QD800 solution consisting of the QD800 bound at the 17th position of the E2 molecules was realized. PL measurements of E2 in the waterish solution let us conclude that the hER_α receptors are immobilized on the silanized surface. The PL signal from the E2-labeled QD800 by using the whole system for a E2 concentration of 1 μM in waterish solution exhibits a high sensitivity which leads to the conclusion that the system could be used for the detection of very low estrogen concentrations in waterish solutions, especially in drinking water.

Acknowledgments

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