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Published in:
Proceedings of the 27th International Conference on Optical Fiber Sensors

Publication date:
2022

Document Version
Peer reviewed version

Towards an Online Polymer Optical Fiber Cortisol Sensor for Aquaculture

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Abstract: Monitoring fish stress is important for optimizing aquaculture production. We present the first steps towards an online all-polymer optical fiber aquaculture cortisol stress hormone sensor.

OCIS codes: (060.2370) Fiber optics sensors, (300.6280) Spectroscopy, fluorescence and luminescence.

1. Introduction

According to data from the Food and Agriculture Organization of the United Nations [1], the seafood production from both capture fisheries and aquaculture is increasing. Interestingly, data also shows that since 2012, seafood has mainly been produced by aquaculture. Careful control of water parameters like oxygen, pH and excreted cortisol stress hormone lead to Feed Conversion Rate (mass of given feed divided by fish mass increase) lowering, better fish welfare, meat quality and reduced pollution.

Kaushik et al. [2] provide an overview of state-of-the-art for cortisol detection. The methods are divided into chromatographic techniques, immunoassays and immunosensors, which are then further subdivided into several more specific methods. For a long time the most sensitive method has been the Radio ImmunoAssay (RIA) method. Mota et al. [3] demonstrated a Limit Of Detection (LOD) of 36 fg/mL in 2017. Today, the lowest LOD we can find is 25.9 fg/mL for the fiber optical lossy mode resonance immunosensor method by Usha et al. [4]. The LOD for chromatographic techniques is around 1 pg/mL [2] and for ordinary immunoassays like Enzyme-Linked ImmunoSorbent Assays (ELISA) around 50 pg/mL [5,6]. Fish cortisol is excreted through the gills to surrounding water and it has been shown that fish and water cortisol concentration levels correlate [5,7]. Aquaculture cortisol concentrations are very low, in the range of 1 pg/mL to a few hundred pg/mL [3,5,8,9]. Measurements in the low end are typically made with RIA [8] and in the high with ELISA [5]. Both techniques require time consuming sampling and subsequent laboratory analysis before a concentration is found.

Optical fiber sensors are attractive because they have unrivaled versatile options for *online* chemical measurements in water over long distances due to the optical detection, which solves many compatibility issues, small size, high sensitivity, reliability and low cost. The idea of using a Glass Optical Fiber (GOF) or Polymer Optical Fiber (POF) in cortisol concentration detection was described for the first time by S. M. Babin in a patent from 2009 [10]. Since then, only one paper on a realized high sensitivity online GOF cortisol immunosensor has been published in 2017 [4] and none on POF cortisol sensing. POFs exhibit considerably higher transmission loss than GOFs (dB/km range vs. dB/m). However, the considerably reduced stiffness of the plastic compared to GOFs ensures they do not break. Another attractive property of POFs is that they are easy to modify chemically for much more reliable assembly [11] and integration of the surface with various sensing chemistries [12,13].

The GOF cortisol sensor by Usha et al. is of high performance, but at the expenses of being bulky, complicated to fabricate, require operation in transmission mode and feed/removal of analyte. In our POF immunosensor design, we use for the first time a far more simple POF, membrane, gel, and luminescent competitive assay based principle, which is still sensitive enough for use in aquaculture monitoring. Because the sensing element is at the fiber tip, it runs with inexpensive instrumentation in reflection mode and measurements are made by just dipping/inserting the sensor in the cortisol containing liquid.

2. Sensor design and cortisol detection mechanism

A sketch of the proposed sensor is shown in Fig. 1. A membrane compartment containing a biochemical assay for optical cortisol concentration detection is attached with adhesive (not shown) to a POF. The competitive assay consists of cortisol antibodies and fluorephore tagged cortisol molecules. The membrane has a Molecular Weight Cut-Off (MWCO) which allows cortisol but not the larger fluorephore tagged cortisol to pass in/out of the compartment. The large antibodies are immobilized in a hydrogel at the fiber distal end. To ensure competition between cortisol and tagged cortisol at the antibodies, this gel is sufficiently open-structured for transport of both molecules. The feasibility of using a UV curable organic hydrogel for this purpose is supported by the cortisol

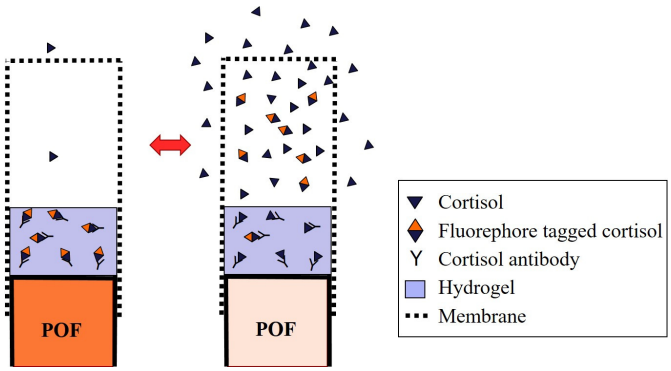

Fig. 1. Sketch showing the basic sensor design elements: POF, fluorephore tagged cortisol, cortisol antibodies, gel for cortisol antibody immobilization at fiber distal end and membrane with MWCO between that of cortisol and tagged cortisol, fixed to POF distal end. Left: Low cortisol concentration where all the tagged cortisol can be captured by cortisol antibodies at the fiber tip. When light is sent through the fiber to excite the fluorephore, this situation leads to high fluorescence pick-up. Right: High ambient cortisol concentration where the antibody captured tagged cortisol at the fiber tip is competed away. When light is sent through the fiber to excite the fluorephore, this situation leads to low fluorescence pick-up because tagged cortisol is competed away from the fiber tip where the incoming light intensity is high.

sensing setup by Zhou et al. [14], who use a less POF compatible inorganic tetramethoxysilane gel for the same purpose. High cortisol concentration competes away the antibody captured tagged cortisol right in front of the fiber and reduces the fluorescence pick-up and vice-versa.

3. Experiments and results

3.1. Fabrication

Tagged cortisol was synthesized from the cortisol derivative cortisol 3-(O-carboxymethyl)oxime (C3CMO) (Product# H6635-50MG, Sigma-Aldrich, Denmark) and Alexa Fluor 488 Hydrazide (AF488H) (Product# 10296832, Fisher Scientific, Denmark) by using the cross-linking agents N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Product# 03449-1G, Sigma-Aldrich, Denmark) and 1-Hydroxy-7-azabenzotriazole (HOAt) (0.6 M in dimethylformamide (DMF), Product# 41996-100ML-F, Sigma-Aldrich, Denmark).

C3CMO and AF488H was chosen for one common reason. The carboxyl group of C3CMO and the hydrazide primary amine on AF488H makes EDC cross-linking possible. Besides, C3CMO is used for raising the antibodies studied in this work, the AF488H exitation and emission wavelengths $\lambda = 493$, $\lambda = 517$ respectively, are in the POF optical transmission range and this fluorephore is very stable in a broad pH range (4-10).

The conjugation procedure used here was as follows. Anhydrous dimethylsulfoxide (*a*DMSO) (Product# 276855-100ML, Sigma-Aldrich, Denmark) was used as solvent for the reactions for three reasons: 1) It dissolves both the hydrophobic C3CMO and the hydrophillic AF488H well, 2) It minimizes hydrolysis side reactions [15], 3) It is a polar aprotic solvent, which increases the AF488H primary amine nucleophilicity in the reaction compared to water based reactions. The role of HOAt is to reduce racemization of the active ester O-acylurea to the inactive N-acylurea during the reaction [16]. The molar mix ratio of the chemicals was C3CMO(4):EDC(2):HOAt(4):AF488H(1). Large excess C3CMO relative to EDC was used to increase the reaction probability, i.e. that almost all EDC will be used so only a little is left to react with the carboxylic acid on AF488H. This also reduce the aforementioned active ester racemization problem [15]. Similarly, a large excess of HOAt relative to the EDC ensures maximum conversion of the C3CMO-EDC active ester to another, which do no turn inactive [16]. The molar amount of this ester can be up to double the amount of AF488H, which again increases reaction probability with the hydrazide primary amine on AF488H. 1 mg AF488H was dissolved in 0.5 mL *a*DMSO in the as supplied vial and placed on a rocking table (BenchRocker™, Benchmark, USA) for at least 10 mins. before use. Stock solutions of C3CMO, EDC, HOAt in *a*DMSO were made with stirring for at least 5 mins. before use: 10 mg C3CMO/1.5 mL, 15 mg EDC/10 mL and 0.5 mL of 0.6 M HOAt/19.92 mL. Then 448 µL EDC solution was added to 458 µL C3CMO solution in a 1.5 mL eppendorf tube and the mixture was placed on the rocking table for 1 min. Next, 477 µL HOAt solution was added and the vial placed on the rocking table for 15 mins. Finaly, this solution was added to the AF488H solution and left to react on the rocking table for 24 hrs.

The C3CMO-AF488H product and remaining reactants/byproducts were clearly identified/separated with Thin

Layer Chromatography (TLC) (20 cm x 20 cm x 0.02 cm silica gel on Al foil, Product# 56524-25EA, Sigma Aldrich, Denmark) using a 80/20/0.1 by volume eluent mixture of isopropanol (IPA), water, triethylamine. DMSO and DMF was removed before elution by evaporation in a vacuum oven at ~0.02 mbar and 45 °C for 30 mins. Retention factors R_f were 0.3 and 0.15 for C3CMO-AF488H, AF488H respectively. Liquid Chromatography - Mass Spectrometry (LC-MS) (Waters ACQUITY UPLC system equipped with SQD2 electrospray MS detector) was used for addtional verification of C3CMO-AF488H synthesis. The C3CMO-AF488H mass 965 D was not directly observed due to fragmentation in the MS. Thus, a strong signal was observed at 869 D corresponding to C3CMO-AF488H with one stripped off SO_4^{2-} group (96 D).

Preparative TLC (PTLC) (20 cm x 20 cm x 0.2 cm silica gel on glass plates, Product# Z513059, Sigma Aldrich, Denmark) was used for the large amount separation of C3CMO-AF488H from remaining reactants/byproducts. A 16 cm long line containing ~200 µL of the crude was dispensed 2 cm from the bottom of each of two plates. DMSO and DMF was then removed by evaporation in a vacuum oven at ~0.02 mbar, 45 °C for 30 mins. This was repeated until ~1 mL crude was dispensed on each plate. Separation was then made using 200 mL of a 160/40 v/v eluent mixture of IPA and 2-(N-morpholino)ethanesulfonic acid buffer (MES) (BupH™ MES Buffered Saline: 0.1 M MES, 0.9 % sodium chloride, pH 4.7. Product# 28390, Fisher Scientific, Denmark). Elution takes 5 to 6 hrs. After overnight drying in a fume-hood, C3CMO-AF488H, AF488H was identified at $R_f = 0.4 - 0.5$ and $R_f = 0.2$ respectively under under 365 nm light illumination. Excess C3CMO and HOAt was identified at $R_f = 0.7$ and $R_f = 0.8$ under 254 nm light illumination. The silica gel containing C3CMO-AF488H was scraped of the glass plates and washed with stirring in 20 mL milliQ water for at least an hour before centrifugation (miniSpin, Buch & Holm, Denmark) in 2 mL vials at 13.4 krpm for 2 mins. The water containing C3CMO-AF488 was pipette collected from the vials. 0.5 mL water was added to the vials, they were vortexed (BenchMixer™, Benchmark, USA) until the silica gel was loose again and then placed on a rocking table overnight. Then the vials were centrifuged again and the C3CMO-AF4888H containing water was again collected. This wash was repeated once more.

The cortisol monoclonal antibodies (mAb) XM210 (Product# GTX21949, Nordic BioSite, Denmark), A29220 (Product# A29220314P, biospacific, Emeryville, CA 94608, USA) and F4P1A3 (Product# LS-C355947, Nordic BioSite, Denmark) were tested to see which one is best for the sensor assay, because they have all been raised using C3CMO conjugation to proteins at the same C3 location as where we make the AF488H conjugation. The mAbs were tested in a setup similar to ELISA. First they were bonded to protein A/G at the bottom of ELISA wells (Reacti-Bind™ Protein A/G Coated Strip Plate, Product# 10073883, Fisher Scientific, Denmark). Then C3CMO-AF488H was bonded to the mAbs. After wash where non-bonded tagged cortisol was removed, the fluorescence intensity was monitored on a Thermo Scientific VarioSkan Lux instrument. Blind tests where unconjugated AF488H was added to the wells were also made. Highest fluorescence signal was observed for mAb XM210, which was therefore selected for further sensor development. Lowest signal was for all mAbs seen from the wells where only unconjugated AF488H was added, which is a third confirmation of the successful synthesis of C3CMO-AF488H. The gel type and synthesis method earlier described by Janting et al. [13] will be used. We selected a cellulose acetate membrane (Product# Z368024-25EA, Sigma-Aldrich, Denmark) with MWCO 500 D, which ensures cortisol ($M_W = 362$ D) can pass through, but not C3CMO-AF488H ($M_W = 965$ D). The first embodiment of the sensor is shown in Fig. 2.

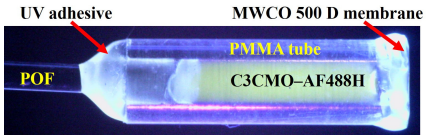

Fig. 2. The first embodiment of the sensor design in Fig. 1. A PMMA plastic tube is attached with UV adhesive (DYMAX 1191-M-VT, Diatom A/S, Denmark) at one end to a multimode PMMA POF (Product# 02534, Edmund Optics®, England) with diameter 1 mm. The tube is filled with gel including immobilized mAb XM210 at the fiber distal end (not included in this picture), C3CMO-AF488H solution and the membrane is glued with the same UV adhesive to the other tube end.

3.2. Characterization

The competitive assay and membrane working principle have so far been tested. The membrane was tested by filling the PMMA tube in Fig. 2 with cortisol or C3CMO-AF488H dissolved in water and dipping the membrane mounted end into water in an eppendorf tube. After different times, drops of water from the eppendorf tubes were placed on TLC plates, and it was confirmed that cortisol can pass through the membrane, but not C3CMO-AF488H. The competitive assay sensor principle was tested by saturating XM210 mAbs grafted to protein A/G

at the bottom of ELISA wells with C3CMO-AF488H, which was then gradually competed away with cortisol solutions of different concentration, see the results in Fig. 3. Each measurement was made by incubating with the specific solution, washing and reading the fluorescence intensity with the Varioskan-Lux instrument.

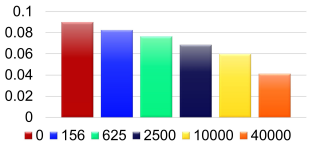

Fig. 3. Sensor assay fluorescence intensity as a function of competing cortisol concentrations from 0 pg/mL (left) to 40,000 pg/mL (right) for the fabricated sensor assay.

4. Conclusion

We conclude, that we have found a working fluorescence based cortisol detection assay consisting of C3CMO-AF488H and mAb XM210, adaptable for POF sensing. Further, we have identified a suitable C3CMO-AF488H/cortisol selective membrane and antibody XM210 immobilization gel for the sensor. Future work will focus on full sensor assembly and characterization.

5. Acknowledgement

This work was supported by The European Maritime and Fisheries Fund and The Danish Fisheries Agency.

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