MIMICKING INSECT SIGNALING: ARTIFICIAL GLAND FOR BIOSYNTHESIS AND RELEASE OF SEMIOCHEMICALS FOR COMMUNICATION

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ABSTRACT

We explore the functionality of a complete chemoemitter platform mimicking the biosynthetic pathways and release of female sex pheromones of the *Spodoptera littoralis* moth. This artificial gland system consists of a microreactor in which the pheromone biosynthesis takes place, and a micromachined evaporator that releases ratiometrically-encoded blend into the environment. The artificial gland was implemented for the investigation and detection of patterns by highly sensitive olfaction system of male moths in electroantennographic and behavioral assays. Thus, a new information emission scheme by utilizing semiochemicals as a data carrier was demonstrated.

INTRODUCTION

Recent advances in microfluidics technology are revolutionizing research dedicated to understand pheromone biosynthesis pathways and the detection of compounds that constitute chemical communication between insects. Until now, the release of chemical stimuli in wind tunnel experiments on insect flight behavior relied mainly on the passive evaporation of volatile chemicals from a lure, usually made from a filter paper or a rubber septum [1], therefore the control of blend composition and emission rates is limited, especially for low concentrations of released volatiles. The temporal modulation of chemical signals in such system is problematic. Recently we have presented a microevaporator capable of transforming liquid pheromones into precise ratios of vapour concentration of volatiles [2]. Here we demonstrate the follow-up step towards an artificial communication system that mimicks biological machinery that allow eusocial insects to exchange information [3]. In a novel approach to information and communication technology, we have developed a chemoemitter system, which comprises a bio-microreactor connected to a microevaporator, capable to produce and release a pre-defined blend of pheromone compounds.

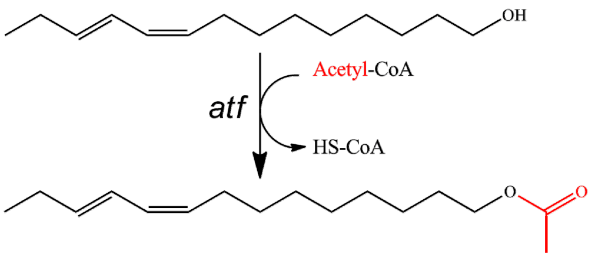

Figure 1: Last step of the pheromone biosynthesis of Spodoptera littoralis.

We report the infochemical production of (*Z,E*)-9,11-tetradecadienyl acetate, the major component of the pheromone of the Egyptian armyworm *S. littoralis* (Lepidoptera: Noctuidae), from the corresponding alcohol by an alcohol acetyl transferase (*atf*) [4], mimicking the last step of the pheromone biosynthesis inside the microreactor (Figure 1).

EXPERIMENTAL

The artificial gland consists of microreactors in which the pheromone biosynthesis takes place, and a micromachined evaporator that releases the eluents into environment (Figure 2).

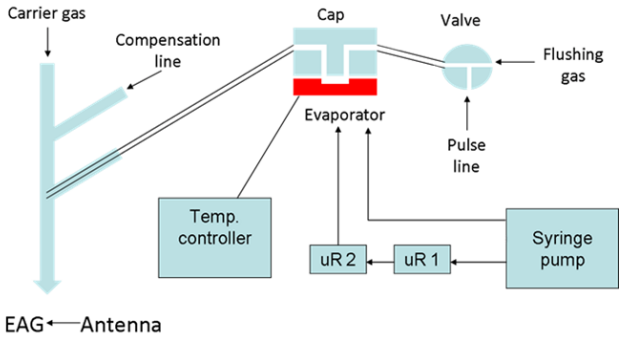

Figure 2: Schematic representation for the integration of two microreactors, evaporator and EAG detection setup.

The major component, (*Z,E*)-9,11-tetradecadienyl acetate ((*Z,E*)-9,11-14:OAc), of the pheromone blend of *S. littoralis* is biosynthesized from the precursor (*Z,E*)-9,11-tetradecadienol ((*Z,E*)-9,11-14:OH) by recombinant alcohol acetyl transferase (*atf*) immobilized inside the microreactor. Nitrilotriacetic acid (NTA) functionalized agarose beads were packed into microreactor, using a procedure reported earlier by *Seong et al* [5], in order to immobilize the purified His_6-tagged *atf*. The microreactor was fabricated from silicon and glass by deep reactive ion etching (DRIE) and anodic wafer bonding (Figure 3).

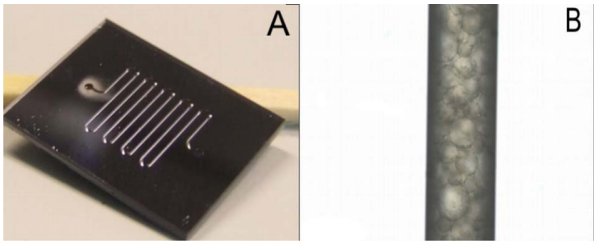

Figure 3: A silicon-glass microreactor with a rectangular channel (W x H) 250 x 200µm, coated with five layers of anti-adsorption PEM (A), image of the NTA functionalized beads entrapped inside the microreactor (B).

A polyelectrolyte multilayer anti-adsorption coating was formed on the microreactor walls to prevent adsorption of the pheromone.

The micromachined evaporator consists of a silicon membrane (5.00 x 5.00 x 0.04 mm) perforated with ~40000 via-holes. Rectangular microfluidic channels deliver the mixture of the pheromones from two inlets to the reservoir (375 nL) located under the membrane. The liquid passes through the membrane and evaporates from small droplets that are formed on the outlet of every via-hole (Figure 4).

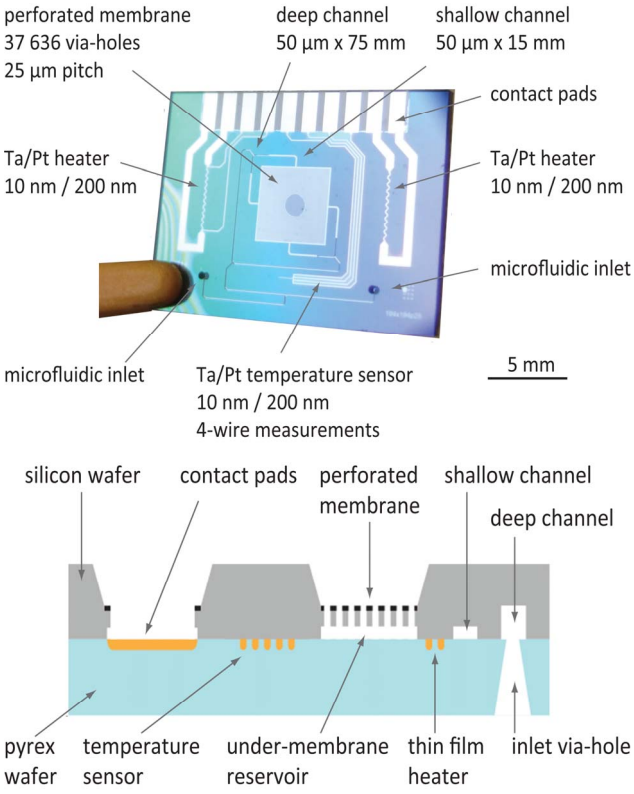

Figure 4: Photograph of the evaporator chip (channel side visible) and schematic representation of the chip cross-section.

Two thin-film platinum heaters and 4-wire resistive temperature sensor are integrated in evaporator and work in a PID loop in order to stabilize the temperature with variation of 30 mK. Integrated heaters dissipate up to 8W of energy elevating the temperature of the membrane up to 250°C.

The efficiency of the microreactor-evaporator system was evaluated by following methods: adsorption of released vapour in Poropak cartridges (Poropak Q(50/80), Supelco, USA), extraction and quantification by GC-MS, electroantennographic assay (EAG) and behavioural experiments in wind tunnel.

RESULTS AND DISCUSSION

Pheromone adsorption and operational stability of the enzyme immobilized on agarose beads was studied. A slight decrease in the activity of *atf* after immobilization and its detoriation in time were detected. The rate of inactivation of the immobilized *atf* was found (K_D=1.02 min^{-1}). It was proven that the enzyme lost its activity 32 hours after immobilization hence it was concluded that *atf* has moderate operational stability after immobilization. Nevertheless, the margins of its operational stability allow for short-term experiments.

Next the enzyme was immobilized inside a microreactor and its activity was measured for different flow rates leading to variation of the residence times inside the microfluidic system. The residence time of the substrate and product were calculated from the volumetric flow rates with the assumption that the flow regime inside the microreactor was plug-flow.

The performance of enzyme microreactor was evaluated at various flow rates to change the reaction time, and the substrate and product contents of the collected fractions were determined by GC-MS (Figure 5). Based on the collected data, a kinetic model was adapted, Michaelis-Menten model for enzymatic reactions, from which apparent K_m of 130 µM was calculated. This value is in close proximity with the experimental K_m (160 µM) for the *atf* in solution; determined, as activity towards different concentrations of (*Z,E*)-9,11-14:OH. The enzymatic reaction reaches equilibrium within a single passage of substrate stream through the microreactor channel at flow-rate 2.5 µL/min.

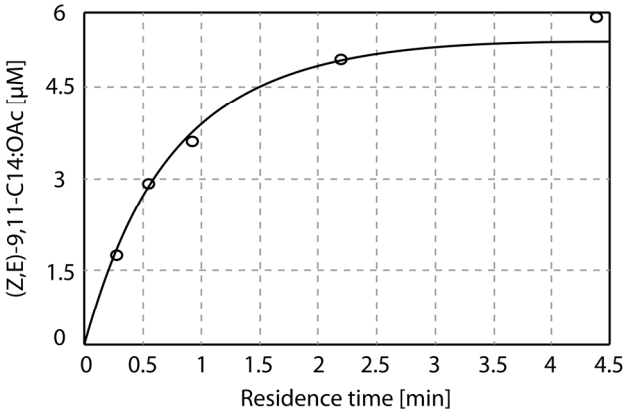

Figure 5: Conversion as a function of time. Product concentrations recovered from two microreactors with identical amount of immobilized atf (10 µg).

To prove the evaporator is capable of releasing a pheromone solution into environment, the dynamic headspace technique was used. The evaporator setup was adapted so that the pheromone vapour was entrapped inside a Poropak cartridge. A PMMA cover with the gas inlet and outlet was attached via PDMS pad to the membrane side of the chip. The temperature was set to 120°C and a 10 µg/µL solution of commercial (*Z,E*)-9,11-14:OAc in hexane was delivered to the chip by syringe pump at a flow rates in the range from 0.5 to 20 µL/min. The gas flow rate was set to 250 mL/min. The collection time varied from 7.5 to 60 min, then the Poropak cartridge was extracted with the hexane and the amount of pheromone collected relative to the collection time was quantified by GC-MS using internal standard. A linear correlation was found between the flow rates and the amount of recovered pheromone, which was larger than 80% of the theoretically released pheromone.

Electroantenography (EAG) technique was used for pheromone detection. The antenna extracted from male *S. littoralis* was fixed on two tungsten electrodes (Figure 6) and stimulus was introduced from the chemoemitter. The depolarization responses of an insect antenna to a chemical stimulus were recorded and quantified [6].

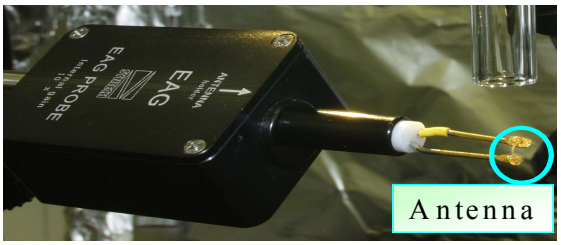

Figure 6: Electroantennographic (EAG) detection setup with antenna extracted from male moth fixed on tungsten electrodes.

The change of electric potential of the male insect antennae is presented in Figure 7.

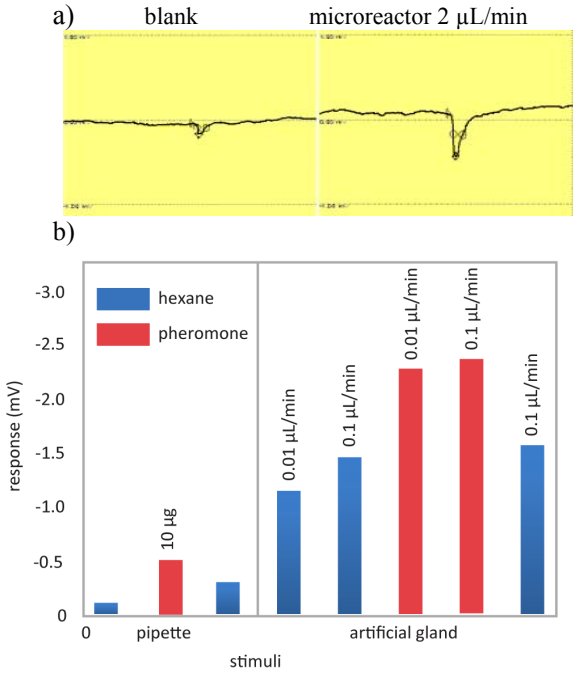

Figure 7: Electroantennographic responses of S.littoralis male antennae exposed to: a) pre-synthesized pheromone, b) stimuli generated by the artificial gland.

The EAG response obtained by using evaporator was much higher in comparison to the classical setup (Pasteur pipette containing a Whatman filter paper with 10 µl of synthetic stimuli (*Z,E*)-9,11-14:OAc adsorbed). Furthermore, the response obtained to the pheromone was significantly higher than the mechanical response (hexane). No difference was found between the injection of 0.01µl/min and 0.1 µl/min of pure pheromone, what suggest that the antenna had reached the saturation level. Since the evaporation rate is limited by the liquid phase delivery the composition of the vapours can be controlled by changing the flow rates of the pheromones pumped into the evaporator. Therefore, the evaporator connected to theoretically unlimited number of microreactors synthesizing the pheromone intermediates, allowed the functional mimicking of any biosynthetic pathway of various species that utilize airborne chemical communication.

The response to the pheromone produced by two microreactors connected in series to increase conversion and the blank (320 µM aq. soln. of (*Z,E*)-9,11-14:OH containing 4% of DMSO), directly introduced into the evaporator from the syringe pump (flow rate = 2 µl/min) without passing through the microreactors, were measured. It was found that antennae depolarization induced by the pheromone emerging from the chemoemitter after partial conversion of alcohol into corresponding acetate inside microreactors and emitted by microevaporator was significantly higher comparing to the response induced by puffs of 10 µg of synthetic pheromone. It was shown that the signal from the chemoemitter could be detected by EAG and differentiated from the blank sample. By varying the flow rate of the precursor, thus changing its residence time inside microreactors, the conversion rate was affected.

Emitted pheromone signals were detected after release from the microreactor-evaporator system in behavioural experiments. The evaporator was placed in the far end of a wind tunnel and *S.littoralis* males were released from a platform at a closer end [7]. A 10 ng/µL aq. soln. of the pheromone containing 4% of DMSO was introduced into the system with a flow rate of 2 µL/min. Insects were subjected to different pheromone sources: the evaporator releasing the pheromone solution as cited, 3 virgin female moths, 10 µg of (*Z,E*)-9,11-14:OAc in hexane on a filter paper, and the evaporator releasing an aq. soln. containing 4% DMSO (no pheromone, blank). The results are presented in Figure 8.

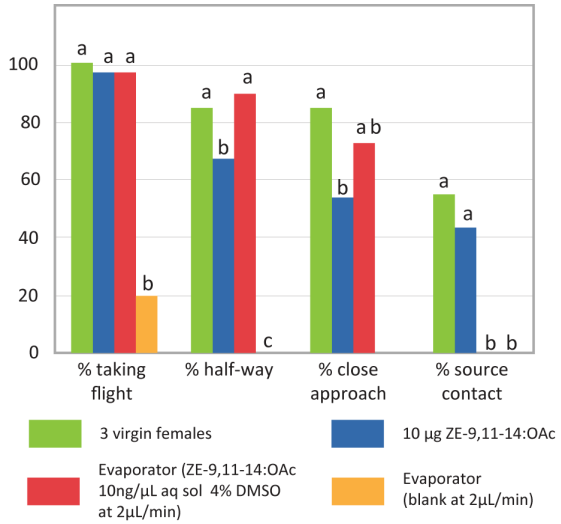

Figure 8: Behavioural response of S. littoralis males (N=20 for each assay) towards different pheromone sources. Same letters corresponding to the same behavior are not significantly different (χ2 test, P<0.05).

The pheromone release from the evaporator elicited a similar attraction as the 3 virgin females in most of the flight parameters considered: 97% vs 100% of insects

taking flight; 90% vs 85% of males arriving to the middle of the tunnel from which 73% vs 85% closely approached the source, respectively. Insects failed, however, to contact the source, most likely because of the working temperature of the evaporator (120ºC). In contrast, during release of the blank only 4 insects (out of 20) took off from the platform, however, neither showed an attractive response nor followed an oriented flight.

In addition, the response of the male moths for very high concentrations of pheromone (0.01 µL/min of pure pheromone) was monitored. Insects have experienced orientation disorder, however elements of behavioural response were observed. Four insects out of five showed antennal response, but failed to either orientate themselves towards the gradient of pheromone concentration or take off. We address this behaviour to the saturation of antenna.

CONCLUSIONS

We have developed an artificial gland, e.g. chemoemitter, consisting of a microreactor and an evaporator, capable to infochemically produce pheromone acetate from its precursor alcohol catalyzed by a biosynthetic enzyme (alcohol acetyl transferase) and release the mixture of both compounds in a controlled manner, thus mimicking the chemical communication in insects. The chemoemitter performance was evaluated by headspace technique, electroantennography and behavioural assay of live insects inside the wind tunnel. The sexual response of male *S. littoralis* moths was triggered by pheromone mixture containing major compound of *S. littoralis* pheromone blend. We envision that this kind of artificial gland leads to development of an artificial communication system based on chemical signaling, in which the ratiometrically-encoded information is transmitted by chemical compounds.

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NOTES

The first two authors contributed equally to this work.

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