Carbohydrate Polymers 100 (2014) 40–45

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A cellulosic responsive "living" membrane

Guokui Qin, Bruce J. Panilaitis*, Zhongyuan Sun David L. Kaplan

Department of Biomedical Engineering, 4 Colby Street, Tufts University, Medford, MA 02155, USA

ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 9 May 2013
Accepted 14 June 2013
Available online 28 June 2013

Keywords:
Living membrane
Bacterial cellulose
Co-cultivation
Gene amplifier
Positive feedback

ABSTRACT

Bacterial cellulose has been demonstrated to be a remarkably versatile biomaterial and widely used in biomedical applications due to its unique physical properties. Here we reported for the first time a "living membrane" system based on recombinant *Escherichia coli* bacterial strains entrapped in cellulosic membranes produced by *Gluconacetobacter xylinus*. Biologically driven detection and identification of a range of target molecules presents unique challenges, and requires that detection methods are developed to be rapid, specific and sensitive. The compatibility of *G. xylinus* and recombinant *E. coli* strains was first investigated for co-cultivation, and the relationship between the number of entrapped *E. coli* and the level of inducible signal achieved was further explored by fluorescent signal observation in confocal microscopy. Finally to amplify the response to inducers for maximum fluorescent signal, a positive-feedback genetic amplifier was designed within recombinant *E. coli* strain entrapped in the living cellulosic membrane system, allowing for the detection mechanism to be extremely sensitive and resulting in a significant fluorescent signal from a single receptor binding event. The living membrane system proposed here will create devices of greater complexity in function for applications in biological and chemical detection.

1. Introduction

Bacterial cellulose has been widely reported in several applications due to its unique physical properties such as higher mechanical strength, water absorption capacity and higher crystallinity, as well as an ultra-fine and highly pure fiber network structure (Charreau, Foresti, & Vazquez, 2012; Thomas, 2008; Yadav, Paniliatis, Shi, Lee, Cebe, & Kaplan, 2010). It has been demonstrated to be a remarkably versatile biomaterial and considered for biomedical applications including wound healing, tissue regeneration, and drug delivery and release (Mayer et al., 1991; Nagai, Nishibe, Makino, Tomimori, & Yamada, 1997; Thomas, 2008). *Gluconacetobacter xylinus* is the most efficient bacterial cellulose producer and has been used as the model system for cellulose biosynthesis (Aloni, Delmer, & Benziman, 1982; Lin, Brown, Cooper, & Delmer, 1985). Typically an extracellular cellulose produced is deposited as a continuous pellicle (membrane) at the gas–liquid interface of a static culture that entraps the bacteria itself and any co-cultured organisms as it grows (Gretz, Folsom, & Brown, 1989). The pellicle is permeable to small protein molecules and its thickness is directly proportional to the duration of cultivation, providing a natural filter for processing of test samples and excellent mechanical properties when compared to cellulose of plant origin. A great number of studies have been completed on the production, physical structure and the chemical composition of bacterial cellulose (Kim, Lee, & Torget, 2001; Lee et al., 2001; Ross et al., 1987; Son, Heo, Kim, & Lee, 2001). For example, the cellulose exhibited strong antiviral activity *in vitro* after chemical modification by sulfation with piperidine *N*-sulfonic acid (Kojima et al., 1991). Several studies have been reported to generate chimerical cellulosic copolymers with non-glucose monomers or containing other polymers, including chitin and chitosan (Kim et al., 2001; Plumbridge, 1995; Yadav et al., 2010). Our most recent work has successfully incorporated heterologous genes into *G. xylinus* with a metabolic engineering-based approach to the rational redesign of cellular metabolites, allowing incorporation of *N*-acetyl-glucosamine (GlcNAc) at much higher levels than previously observed (Yadav et al., 2010; Yadav, Panilaitis, Shi, Numuta, Lee, & Kaplan, 2011). The resulting cellulose-chitin copolymer was demonstrated to be *in vivo* degradable, providing a new route to overcome the longstanding limitation associated with the poor degradability of cellulose for biomedical and biomass conversion applications.

Current detection of target molecules including pathogen and chemical detection often utilizes sophisticated equipment that is fragile and requires significant power supply, and dedicated laboratory space that is not available in all locations (Chepurnykh & Avkhimenko, 1994; Ligon, 2006; Upadhyayula, 2012). A rapid, simple, and low cost detection technology with high sensitivity is needed for stability and functionality at extremes of temperature, humidity and in the absence of elaborate support equipment. Bacteria naturally respond to a wide range of stimuli in their environment. Most of those systems are designed to respond to

* Corresponding author. Tel.: +1 617 627 3607; fax: +1 617 627 3231.
E-mail address: bruce.panilaitis@tufts.edu (B.J. Panilaitis).

0144-8617/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.carbpol.2013.06.019

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant features or description	Source and purpose
Strains		
G. xylinus ATCC 10245	Wild type (wt); standard cellulose production	ATCC; this study
E. coli NEB5α	Gene cloning	NEB; cloning
E. coli BLR (DE3)	High protein expression	Novagen; expression
E. coli BL21star (DE3)	High protein expression	Invitrogen; expression
Plasmids		
pGFPuv	Amp^r; green fluorescent protein (GFP) gene	Clontech; GFP expression
pJexpress-RFP	Kan^r; red fluorescent protein (RFP) gene	DNA2.0; RFP expression
pGEM-T	Amp^r; PCR cloning vector	Promega; cloning
pGEMT-GFP/Lux	Amp^r; recombinant plasmid harboring GFP/lux gene	This study
pGN68	Cam^r; recombinant plasmid harboring GFP/lux gene with *lux* promoter	Nistala et al. (2010)

Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Cam^r, chloramphenicol resistance.

small molecules related to nutrient concentration, quorum sensing, or related functions (Dark, Dean, & Warhurst, 2009). For example, Nistala et al. constructed a modular genetic amplifier in *Escherichia coli* for a one-component tetracycline sensor and a two-component aspartate sensor with the increased sensitivity, based on a constitutively active, autoinducer-independent variant of the quorum-sensing regulator LuxR from *Vibrio fischeri* (Nistala, Wu, Rao, & Bhalerao, 2010).

Given the need to respond to larger molecules in the case of some detection applications, here we developed a "living membrane" system based on recombinant bacterial strains entrapped in cellulosic membranes, making them resistant to environmental degradation and persistent for short or long periods of time. Based on the reporter genes available, we have coupled the natural detection capabilities in bacteria with the expression of specific fluorescent proteins to eventually generate a suite of living detectors for the rapid and specific identification of relevant target molecules. Herein we attempt to engineer a genetic amplifier in *E. coli* to amplify a transcriptional fluorescence signal output entrapped in cellulosic membranes, based on the quorum-sensing regulator LuxR as reported previously (Nistala et al., 2010). The proposed system will be durable and easy to use, requiring no additional power or sophisticated support equipment.

2. Materials and methods

2.1. Bacterial strains, culture media and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. The cellulose producing bacterium *G. xylinus* strain 10245 was obtained from ATCC (Manassas, VA) and cultured in Hestrin and Schramm (HS) medium (0.5% yeast extract, 0.5% peptone, 0.27% Na_2HPO_4, 0.15% citric acid, pH 4.5) supplemented with 2% glucose and grown at 30 °C shaking at 200 rpm or statically. All of *E. coli* cells were cultured in Luria-Bertani (LB) broth at 37 °C unless noted otherwise. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol at 20 μg/mlm and kanamycin at 50 μg/ml. Primers were purchased from Invitrogen Inc. (Carlsbad, CA). Restriction enzymes were purchased from New England Biolabs Inc. (Ipswitch, MA) and used according to the manufacturer's recommendations. All cloning steps were performed in *E. coli* strain NEB5α (New England Biolabs, Ipswitch, MA). Subsequent experiments involving the protein expression under IPTG induction were conducted in *E. coli* strain BL21 star (DE3) (Invitrogen, Carlsbad, CA) and strain BLR (DE3) (Novagen, Madison, WI).

2.2. Plasmid construction and transformation

The plasmid pGN68 with the chloramphenicol selection gene (Nistala et al., 2010) (donated by Prof. Kaustubh Bhalerao, University of Illinois at Urbana-Champaign, USA) was made for the *luxI-GFP-LuxR* transcriptional fusion with the *luxI* promoter. The GFP–LuxR fusion of the GFP and LuxR genes was first PCR amplified using the plasmid pGN68 as the template with the primers GN10F (GGG GAA TTC ATA CGT ATT TAA ATC AGG AGT GGA AAT GAG TAA AGG AGA AGA ACT T) and KW171R (AAT AGC GGC CGC TTA TTA ATT TTT AAA GTA TGG GC) (Nistala et al., 2010). The PCR program was used as follows: 95 °C for 4 min (1 cycle); 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1.5 min (30 cycles) and 72 °C for 10 min (1 cycle). The resulting amplified DNA fragments of approximately 1 kb were gel extracted (Qiagen, Valencia, CA) and then cloned into the *BamH*I and *Not*I restriction sites of the vector pGEM-T (Promega, Madison, WI), yielding the new plasmid pGEMT-GFP/LuxR with the *T7* promoter. Ligation reactions were done using T4 DNA ligase (Invitrogen, Carlsbad, CA) at 16 °C. The ligation product was used to transform *E. coli* DH5α strain (Invitrogen, Carlsbad CA) and successful transformants were identified by plating and incubation on a LB/agar plate containing 100 μg/ml ampicillin. The presence of correct inserts in each construct was identified by colony PCR and confirmed by DNA sequencing.

The plasmid pGFPuv (Clontech, Mountain View, CA) with the ampicillin selection gene was transformed into *E. coli* BLR (DE3) for the expression of green fluorescent protein (GFP). The plasmid pJexpress-RFP (DNA2.0, Menlo Park, CA) with the kanamycin selection gene was transformed into *E. coli* strain BL21 star (DE3) for the expression of red fluorescent protein (RFP). The new plasmid pGEMT-GFP/Lux with the ampicillin selection gene was also transformed into *E. coli* BL21 star (DE3) for GFP expression. A genetic amplifier with the plasmid pGN68 and plasmid pGEMT-GFP/Lux was further transformed into the same *E. coli* BL21 star (DE3) for GFP signal detection.

2.3. Cell growth, cellulose production, and fluorescent protein expression

For cellulose production in static culture, *G. xylinus* strain 10245 cells were grown in 6-well culture plates containing 10 ml of HS medium each well at 30 °C for 1 week. The cellulose pellicles were purified by treating twice with 4% SDS solution at 70 °C for 4 h and 4% NaOH at 70 °C for 4 h to remove the entrapped bacteria, followed by several washes with deionized water as reported previously (Yadav et al., 2010). The purified cellulose mats were further dried at 70 °C for 30 h for next studies. To develop a "living" membrane system with fluorescent signals, *G. xylinus* strain 10245 cells were grown in 6-well culture plates at the same conditions for 4 days, and 2% (v/v) *E. coli* cells harboring the plasmids were then applied into each well and co-cultured with *G. xylinus* cells at 30 °C for 8–12 h. Finally fluorescent protein expression was induced by adding 1 mM ITPG (isopropyl β-D-thiogalactoside) (Fisher Scientific, Hampton, NH) into each well of plates and measured after