Microfabricated silicon nitride membranes for hepatocyte sandwich culture

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ABSTRACT

We have developed a hepatocyte sandwich culture with improved mass transport properties based on ultra-thin microfabricated porous silicon nitride (Si3N4) membranes. The dimensions and uniformity of the membrane pores can be configurable, which confers more control over the mass transport. Instead of collagen gels used in conventional sandwich culture, we utilized galactose ligands immobilized on the Si3N4 membranes to support hepatocyte attachment and function in the sandwich culture. Diffusion studies using FITC-dextrans confirmed that mass transport of the microfabricated Si3N4 membrane based sandwich was significantly better than conventional collagen gel sandwich and can be configured by varying the porosity of the Si3N4 membrane. Hepatocytes cultured in the microfabricated Si3N4 membrane based sandwich culture exhibited earlier apical repolarization and biliary excretion, improved differentiated functions and enhanced drug sensitivity compared to hepatocytes cultured in a collagen gel sandwich. The Si3N4 membrane based sandwich culture allows for a systematic optimization of the mass transport properties of hepatocyte culture by changing the pore size and inter-pore distance. This will enable more effective drug testing applications where optimal mass transport is required for hepatocyte function maintenance and drug accessibility.

1. Introduction

Hepatocytes, the major cell type in the liver, are a crucial component of cell-based liver devices, such as bioartificial liver devices, and in vitro drug testing platforms [1,2]. Cultured primary hepatocytes quickly lose their differentiated functions in vitro due to the absence of an in vivo microenvironment [3]. Several culture methods have been proposed to overcome the loss of cell functionality [4–9]. The collagen gel sandwich is one of the more established culture methods where a hepatocyte monolayer is enclosed between two layers of collagen gel closely mimicking the in vivo microenvironment [10]. This model reestablishes hepatocyte polarity and maintains liver-specific functions for up to 6 weeks [11] and has been used for drug metabolism/toxicity testing [12–15]. However, the accuracy of drug testing may be affected by the use of collagen gels, which have been shown to exhibit batch variation [16], non-uniformity in the gel thickness, and retention of drugs and their metabolites [17,18]. More importantly, collagen gels present a transport barrier for nutrients or metabolic wastes, which affects the functionality of the metabolically-active hepatocytes [19]. The mass transport distance in the collagen gel sandwich is typically 100–200 μm [20], which is greater than the mass transport distance in vivo where the distance from sinusoid to hepatocytes is 5 μm [21]. While there are efforts to improve the mass transport in sandwich cultures by replacing the coated collagen gel with synthetic cell-attachment ligands immobilized on a membrane [22], the mass transport properties of the membrane itself still need to be optimized.

We have developed a microfabricated porous silicon nitride (Si3N4) membrane for sandwich culture of primary hepatocytes.
with improved and configurable mass transport. This is achieved by: (1) the use of a microfabricated silicon nitride (Si$_3$N$_4$) membrane with well defined pore size and inter-pore distance and (2) the replacement of collagen gels with galactose ligands. The microfabricated Si$_3$N$_4$ membrane based sandwich culture (Si$_3$N$_4$-SC) consists of hepatocytes sandwiched between a 1.5 µm thick porous Si$_3$N$_4$ membrane (the top support) and an about 100 µm thick polyethylene terephthalate (PET) film (the bottom support), both immobilized with galactose ligands. The galactose ligands provide functional support for the hepatocytes via asialoglycoprotein receptor (ASGPR) interactions without impeding mass transport [23–25]. Hence, the mass transport in this Si$_3$N$_4$-SC can be mainly configured by changing the porosity of the Si$_3$N$_4$ membrane itself. Hepatocytes in the Si$_3$N$_4$-SC showed earlier apical repolarization and bile excretion, improved differentiated functions and enhanced drug sensitivity compared to hepatocytes cultured in a collagen gel sandwich culture. The capability of the Si$_3$N$_4$-SC to systematically configure mass transport for optimal cell functions and accessibility of xenobiotics is particularly important for effective drug testing applications.

2. Materials and methods

2.1. Materials

Biaxially oriented PET films of 100 µm thickness were purchased from Goodfellow Inc. (Cambridge, UK). AZ7220 photoresist and AZ300MK were purchased from AZ electronic materials USA Corp. (Branchburg, NJ, USA), and 50% glutaraldehyde solution was purchased from MERCK (Singapore). The galactose ligand, 1-O-(6'-aminohexyl)-α-galactopyranoside (AHG, M.W. 279) was synthesized according to the method reported previously [24,26] and verified by NMR spectrum. All other chemicals were purchased from Sigma–Aldrich (Singapore) unless otherwise stated.

2.2. Fabrication of porous Si$_3$N$_4$ membranes

The steps of fabrication for porous Si$_3$N$_4$ membranes are shown in Fig. 1A. Silicon wafer measuring 4 inch was cleaned with piranha solution (3:1 v/v of concentrated H$_2$SO$_4$ to H$_2$O$_2$) at 120 °C for 30 min, and deposited with 100 nm of silicon oxide (SiO$_2$) and 3 µm of Si$_3$N$_4$ by low-pressure chemical vapor deposition (LPCVD). The wafer was spin-coated with AZ7220 photoresist (EVG 101 Advanced Spray Coating System, St. Florian am Inn, Austria), exposed under UV light for 5 s by EVG620 manual precision alignment system and developed in AZ300MK. The developed features were etched by ICP Dielectric Etching System (Adixen AMS 100, Annecy, France). The residue of AZ7220 photoresist was removed by immersing wafers in N-methylpyrrolidone (NMP) solution and sonicating them at 70 °C for 30 min. After rinsing with deionized (DI) water and drying with nitrogen, the same process was repeated on the other side of the wafer. Finally, the porous Si$_3$N$_4$ membranes were released from the wafer by etching with tetramethylammonium hydroxide (TMAH) at 90 °C for 7–8 h [27], which etches the silicon around the Si$_3$N$_4$.

2.3. Immobilization of galactose ligand on porous Si$_3$N$_4$ membrane

The porous Si$_3$N$_4$ membrane surface was immobilized with galactose ligands as described previously with modifications [28,29]. The untreated membranes were immersed in a piranha solution at 120 °C for 30 min, rinsed with DI water and dried with nitrogen. They were immersed in 5% 3-aminopropyltriethoxysilane (APTES) solution in absolute ethanol for 2 h. Samples were rinsed with ethanol and dried with nitrogen. They were then exposed to 2.5% glutaraldehyde solution in PBS for 1 h at room temperature. The glutaraldehyde solution was removed and the samples were exposed to 2 mg/ml galactose solution until saturated. The samples were quenched with 0.5% ethanolamine solution for 15 min and washed with PBS.

Fig. 1. Fabrication and characterization of porous Si$_3$N$_4$ membrane. (A) flowchart of porous Si$_3$N$_4$ membrane fabrication; (B–D) characterization of porous Si$_3$N$_4$ membrane by scanning electron microscopy (SEM); (B) SEM image at low magnification showing a large region of the membrane; (C) SEM image at high magnification showing the pore size (20 µm) and inter-pore distance (20 µm); (D) SEM image at high magnification showing the thickness of membrane (1.5 µm).
2.4. Characterization of porous Si3N4 membrane immobilized with galactose ligand

2.4.1. Wettability of porous Si3N4 membrane by static water contact angle measurement

To verify the functionalization of the porous Si3N4 membrane surface and to examine the changes in surface wettability at different stages during the immobilization of galactose ligand on the porous Si3N4 membrane, static water contact angle measurement was performed [30].

2.4.2. Determination of surface amino group concentration

The density of surface amino groups was determined by using colorimetric Acid Orange II assay as previously reported [31]. Samples were immersed in 500 μg Acid Orange II solution in distilled water at pH 3 (diluted HCl) to allow for protonation of amines. After shaking overnight at room temperature, the samples were washed twice with water at pH 3 (diluted HCl). The samples were immersed in distilled water at pH 12 (diluted NaOH) and shaken at room temperature for 15 min. The optical density of the solution was measured spectrophotometrically at 492 nm by a microplate reader (Tecan Safire®).

2.4.3. Surface roughness measurement of galactose-immobilized porous Si3N4 membrane

Changes in surface roughness of the porous Si3N4 membrane before and after galactose immobilization were measured by atomic force microscopy (AFM) (Veeco AFM, USA) [32].

2.4.4. Determination of surface galactose ligand concentration

A galactose-specific binding lectin labeled with Agglutinin RCA120 peroxidase was used for the semi-quantification of surface galactose ligand on porous Si3N4 membrane. Membranes were rinsed twice with PBS containing 2% (v/v) Tween-20 for 2 min at room temperature to block non-specific binding sites, followed by rinsing the membranes twice with PBS. A 100 μl of RCA120 dissolved in PBS (1 μg/ml w/w) containing 0.05% (v/v) Tween-20 was then added and incubated for 2 h at 37°C. The unbound lectin fraction was removed and samples were washed with PBS. Surface bound lectin was dissociated by adding 100 μl of supersaturated galactose solution (50 mg/ml) and incubated for 2 h at 37°C. The peroxidase activity in the solution was initiated by adding 100 μl of standard tetramethyl benzidine (TMB) substrate and quenched by adding 100 μl of 4 N HCl. The absorption of the TMB was measured by spectrophotometry at 652 nm. The density of surface amino groups was quantified from an Acid Orange II standard curve.

2.5. Fabrication of PET film immobilized with galactose ligand

PET film immobilized with galactose ligand was fabricated as reported previously [33]. It was cut into 12 mm diameter circular disks to fit the in-house designed holder.

2.6. FITC-dextran diffusion measurement

The mass transport properties of two Si3N4-SCs were compared to a collagen gel sandwich culture by measuring the diffusivity of fluorescein isothiocyanate-conjugated dextran (FITC-dextran with molecular masses: 9.5 kDa, 70 kDa and 150 kDa) through the top membrane of the respective sandwich cultures in a donor–receptor compartmental model with slight modification [34,35]. The top membrane of the Si3N4-SC was two galactose-immobilized porous Si3N4 membranes with 20 pores spaced either 5 μm or 20 μm apart, while the top membrane of the collagen gel sandwich was a collagen gel coated polycarbonate (PC) membrane. The top membrane was clamped between the receptor and donor compartments using in-house designed holders. Donor compartments were filled with 0.2% FITC-dextran in PBS whereas receptor compartments were filled with 1 ml of PBS. Solutions were taken from the receptor compartment at specific time points and replaced with fresh PBS. The concentrations of FITC-dextran were measured at 490 nm excitation/525 nm emission against FITC-dextran standards using the Tecan microplate reader (Tecan Safire®).

2.7. Primary rat hepatocyte isolation and culture

Hepatocytes were harvested from male Wistar rats weighing 250–300 g by a two-step in situ collagenase perfusion method [36]. Animals were handled according to the IACUC protocol approved by the IACUC committee of the National University of Singapore. Viability of the hepatocytes was determined to be ~85% by Trypan Blue exclusion assay. Freshly isolated hepatocytes were seeded onto collagen-coated or galactose-immobilized substrates at 1.5 × 10^4 cells/cm² in a 24-well plate and cultured in Williams’ E medium supplemented with 10 ng/ml HEPES, 1 mg/ml BSA, 10 ng/ml EGF, 0.5 μg/ml insulin, 5 μg/ml dexamethasone, 50 ng/ml linoleic acid, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated with 5% CO₂ at 37°C and 95% humidity for 24 h. A second substrate, either collagen-coated PC membrane or galactose-immobilized porous Si3N4 membrane (20 μm pore size and 20 μm inter-pore distance, corresponding to 20% porosity, the same as that of PC membrane), was placed on top of the cells on the first substrate forming a sandwich culture.

2.8. Scanning electron microscopy (SEM)

Hepatocytes were fixed with 2.5% glutaraldehyde at room temperature for 1 h and post-fixed with 1% osmium tetroxide at room temperature for 1 h. The cells were dehydrated using an ethanol series (25%, 50%, 75%, 95% and 100%) before undergoing critical point drying for 2 h in absolute ethanol. The samples were then sputter-coated with platinum (FC-1600, JEOL, Tokyo, Japan) and viewed under a field-emission scanning electron microscope (JSM-7400F, JEOL, Tokyo, Japan).

2.9. Assessment of hepatocyte polarity and functions

2.9.1. Bilary excretion visualized by fluorescein

To visualize the biliary excretion, hepatocytes sandwich cultures were incubated with 15 μg/ml fluorescein diacetate (FDA) (Molecular probes, Oregon) in Williams’ E medium at 37°C for 45 min under darkness. The cells were rinsed with PBS and fixed with 3.7% paraformaldehyde (PFA) at 37°C for 15 min and viewed under a confocal microscope using a 40× objective lens (Fluoview 300, Olympus, Japan). Fluorescein localized in the inter-cellular area was considered excreted FDA and was expressed as a ratio to intra-cellular FDA by image quantification (Image-Pro Plus, Media Cybernetics, USA) [22].

2.9.2. Immunostaining of Mrp2 and CD147

Immunostaining of the apical multidrug resistance protein, Mrp2, and the basolateral protein, CD147, was performed to investigate the repolarization of hepatocytes in Si3N4-SC and collagen gel sandwich cultures. Cells were fixed with 3.7% PFA at 3°C for 15 min and blocked with 10% FCS in PBS at room temperature for 1 h. Then the cells were incubated with the primary anti-Mrp2 rabbit monoclonal antibody and primary anti-CD147 mouse monoclonal antibody (Serotec Inc. Raleigh, NC) overnight at 4°C. After three times rinsing with PBS, the cells were then incubated with the secondary FITC-conjugated goat anti-rabbit IgG and secondary TRITC-conjugated goat anti-mouse IgG at room temperature for 1 h. The cells were rinsed with PBS three times, placed in fluorescent mounting medium (Dako, Denmark) and viewed under a confocal microscope with 40× water lens (Fluoview 300, Olympus, Japan). Image processing was performed to quantify the extent of apical localization of Mrp2 as an indicator for polarity reestablishment. Images were filtered for the Mrp2 signals located at inter-cellular area by a threshold filter. The area of inter-cellular Mrp2 (Acell) was expressed as the ratio of the total Mrp2 and CD147 areas (Acell) = Acell/Atotal × 100%.

2.9.3. Measurement of liver-specific functions

The urea production of hepatocytes incubated with 2 mM NH₄Cl in culture medium for 90 min was measured using the urea nitrogen kit (Stabino Laboratory, Boerne, TX). The daily albumin synthesis was measured using the rat albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA). The cytochrome P450 (CYP) 1A1/2 enzyme activities were evaluated by 7-ethoxyresorufin-O-deethyllysis (EROD) assay, which was performed by incubating the hepatocytes with 39.2 μM 7-ethoxyresorufin in culture medium at 37°C for 4 h. The quantity of resorufin produced was measured using the Tecan microplate reader at 543 nm excitation/570 nm emission against resorufin standards.

2.10. Acetaminophen-induced hepatotoxicity

Hepatocytes cultured in the Si3N4-SC and collagen gel sandwich were treated with acetaminophen (APAP) to assess their differential responses to drug-induced hepatotoxicity, and hepatocytes cultured on the collagen-coated coverslip surfaces were used as the negative control. APAP stock solution dissolved in dimethylsulfoxide (DMSO) was diluted with culture medium to 10 μM or 40 μM and the final concentration of DMSO in the medium was kept at less than 1%. Hepatocytes in the two sandwich configurations and on the collagen-coated coverslip were exposed to APAP for 6 h and the cell viability was measured by MTS assay using the CellTiter 96 Aqueous One Solution Reagent (Promega, USA).

2.11. Statistical methods

Data from 2–4 independent experiments were analyzed and values were represented as mean ± standard error of the means (s.e.m.). The Student’s t-test was used to analyze the statistical significance of specific pairs of the data. Values with a p value less than 0.05 were considered statistically significant.

3. Results

3.1. Fabrication and characterization of porous Si3N4 membrane

Microfabricated porous Si3N4 membranes (Fig. 1A) were characterized by scanning electron microscopy (SEM) (Fig. 1B–D). The porosity of the Si3N4 membrane is determined by the pore size and inter-pore distance, which can be precisely controlled by microfabrication. The porosity is calculated by dividing the porous area
by the whole area of the membrane. Fig. 1C illustrates a porous Si$_3$N$_4$ membrane with a pore size of 20 μm in diameter and an inter-pore distance of 20 μm, corresponding to a porosity of 20%. The porous Si$_3$N$_4$ membrane was 1.5 ± 0.16 μm thick (Fig. 1D) and had the same porosity as that of the commercially available PC membrane that was used as a control.

3.2. Characterization of galactose-immobilized porous Si$_3$N$_4$ membrane

Galactose ligands were immobilized on the porous Si$_3$N$_4$ membrane by solution based silanization [28,29]. The wettability of the porous Si$_3$N$_4$ membrane changed during different stages of the galactose immobilization process and were in concordance with reported values (Fig. 2A) [32,37]. To optimize the silanization process, we quantified the amount of amino groups on the surface obtained at different concentrations of APTES (Fig. 2B). The amount of amino groups increases with APTES concentration and saturates at 0.04 μmol/cm$^2$ at 5% APTES. We used 5% APTES, since excessive APTES will self-polymerize, resulting in non-uniformity of the membrane surface [38]. Closer examination using atomic force microscopy showed that the surface roughness of the porous Si$_3$N$_4$ membrane increased from ~10 nm before galactose immobilization to ~65 nm after the galactose immobilization (Fig. 2C). Quantification of the amount of galactose ligands by lectin assay showed that the amount of galactose after immobilization was seven times higher than those on the unmodified surface (Fig. 2D). The signals on the untreated surfaces are a result of non-specific binding of lectin.

3.3. Improved and configurable mass transport in Si$_3$N$_4$-SC

The Si$_3$N$_4$-SC was developed with the aim of improving its mass transport over conventional collagen gel sandwich. This can be achieved by optimizing the porosity of the Si$_3$N$_4$ membrane. One way to change the porosity of the Si$_3$N$_4$ membrane is to vary the inter-pore distance between the 20 μm pores, in this case, from 20 μm to 40 μm.
20 μm (corresponding to 20% porosity) to 5 μm (corresponding to 50% porosity). To quantify the mass transport properties, FITC-dextrans of different molecular masses were diffused through three different sandwich configurations of the following top membranes: (1) microfabricated galactose-immobilized Si$_3$N$_4$ membranes (50% porosity), (2) microfabricated galactose-immobilized Si$_3$N$_4$ membranes (20% porosity) and (3) collagen-coated commercially available PC membranes (20% porosity) (Fig. 3). Diffusion of low molecular mass (9.5 kDa) FITC-dextrans through all three membranes was comparable (Fig. 3A). At 70 kDa, FITC-dextrans have the highest diffusion through Si$_3$N$_4$ membranes with 50% porosity, followed by Si$_3$N$_4$ membranes with 20% porosity and the PC membrane (Fig. 3B). At 150 kDa, FITC-dextran exhibits similarly low diffusion through the PC and the Si$_3$N$_4$ membranes with 20% porosity while through the Si$_3$N$_4$ membranes with 50% porosity there was a significantly improved diffusion (Fig. 3C). These results indicate that the mass transport via our galactose-immobilized porous Si$_3$N$_4$ membranes is improved over collagen-coated PC membranes and that mass transport can be configured by varying the pore size and inter-pore distance of membranes.

3.4. Morphological stabilization of hepatocytes in Si$_3$N$_4$-SC

Although galactose-immobilized substrates are commonly used for spheroid cultures of hepatocytes, it has been reported that hepatocytes’ morphology on galactose-immobilized bioactive substrates is unstable [24,33]. To investigate whether the application of the galactose-immobilized Si$_3$N$_4$ membranes in a sandwich culture configuration has any effect on hepatocytes morphology, we closely examined the morphology of primary hepatocytes cultured on galactose-immobilized PET film, galactose-immobilized porous Si$_3$N$_4$ membrane and in the Si$_3$N$_4$-SC (galactose-immobilized PET film as bottom support and galactose-immobilized porous Si$_3$N$_4$ membrane as top support) by using scanning electron microscopy (Fig. 4). Within 2 days after seeding, hepatocytes attached and spread well on all culture configurations (Fig. 4A,D,G). On day 4, multi-cellular aggregates formed on both the galactose-immobilized PET film and the galactose-immobilized porous Si$_3$N$_4$ membrane (Fig. 4B–F), with a large number of the aggregates detaching from the substrates on day 6. However, in our Si$_3$N$_4$-SC, hepatocytes maintained a stable morphology throughout the 6 days of culture (Fig. 4G–I).

3.5. Earlier polarity reestablishment and higher functions of hepatocytes in Si$_3$N$_4$-SC

Hepatocytes are polarized cells with basolateral domains at the cell–matrix interaction area and apical domains at the cell–cell interaction area [39]. Directed transport of metabolites from the basolateral domain to the apical domain constitutes an important hepatocyte function. To compare the dynamics of hepatocyte polarity reestablishment in the Si$_3$N$_4$-SC with that of the collagen gel sandwich culture, we investigated the biliary excretory function of hepatocytes by incubating the hepatocytes with fluorescein.

Fig. 3. Mass transport of Si$_3$N$_4$-SC can be configured by varying the porosity of the Si$_3$N$_4$ membrane as demonstrated by diffusion of FITC-dextrans of different molecular masses: (A) quantification of 9.5 kDa FITC-dextran diffusion; (B) quantification of 70 kDa FITC-dextran diffusion; (C) quantification of 150 kDa FITC-dextran diffusion. (□) microfabricated Si$_3$N$_4$ membrane of 20 μm of pore size and 5 μm of inter-pore distance (50% porosity); (■) microfabricated Si$_3$N$_4$ membrane of 20 μm of pore size and 20 μm of inter-pore distance (20% porosity); (▲) collagen-coated PC membrane (20% porosity). (Data are mean ± s.e.m. of four independent experiments; (*) paired student t-test between Si$_3$N$_4$ membrane with 20% porosity and PC membrane, p < 0.05; (#) paired student t-test between Si$_3$N$_4$ membrane with 20% porosity and Si$_3$N$_4$ membrane with 50% porosity, p < 0.05.)
diacetate (FDA), a non-fluorescent precursor which is hydrolyzed by intra-cellular esterases into fluorescein and excreted into bile canaliculi. On day 1 of culture, hepatocytes in the Si3N4-SC exhibited subtle concentrated localization of fluorescein in the apical domain between adjacent hepatocytes (arrow in Fig. 5D), while fluorescein in the collagen gel sandwich culture was evenly distributed in the intra-cellular space (Fig. 5A). On day 2 of culture, hepatocytes in both sandwich cultures showed more concentrated localization of fluorescein than on day 1 (Fig. 5B,E). The concentration of excreted fluorescein at the apical domain further increased on day 3 (Fig. 5C,F) and maintained at the same level until the end of 1 week (data not shown). The fluorescein localized in the inter-cellular area between hepatocytes was quantified by image processing (Fig. 5).

Furthermore, we investigated the expression of Mrp2, one of the bile canalicular transporters at the apical domain, and CD147, a basolateral domain protein by double immunostaining (Fig. 6). On day 2, the hepatocytes in our Si3N4-SC showed a co-localization (yellow) of Mrp2 (green) with CD147 (red) (Fig. 6D), while Mrp2 remained diffusively distributed within the cells in the collagen gel sandwich (Fig. 6A). On day 4, the apical Mrp2 became concentrated along cell–cell contacts (arrows in Fig. 6E) and this concentrated distribution was maintained until day 6 in the Si3N4-SC (arrows in Fig. 6F), whereas in collagen gel sandwich the concentrated distribution of Mrp2 along cell–cell contacts was not observed until day 6 (arrows in Fig. 6C). Mrp2 located in the apical domain was quantified by image processing (Fig. 6). Together, Figs. 5 and 6 indicate that the hepatocytes in Si3N4-SC established apical repolarization earlier than those in collagen gel sandwich, which is very important for excreting metabolites and toxins from the body [40] and critical for drug discovery and development [41].

We then compared the synthetic and metabolic functions of hepatocytes in the Si3N4-SC with those in the collagen gel sandwich. Albumin secretion was similar in both culture configurations (Fig. 7B), while urea production and CYP1A1/2 enzymatic activity of hepatocytes in Si3N4-SC were significantly higher than those in the collagen gel sandwich (Fig. 7A,C). These results show that hepatocytes cultured in Si3N4-SC have comparable or improved functions over the hepatocytes cultured in collagen sandwich.

3.6. Higher drug sensitivity of hepatocytes in Si3N4-SC

CYP450 enzymes, including CYP1A1/2, are important for drug testing as they metabolize many endogenous substrates as well as xenobiotic agents such as acetaminophen (APAP). Since hepatocytes cultured in the Si3N4-SC exhibited higher resorufin production in Fig. 7 due to either enhanced CYP1A1/2 enzymatic activity or improved access to assay substrate (7-ethoxyresorufin), the Si3N4-SC may be more sensitive to drug mediated hepatotoxicity as a result of enhanced CYP450 enzymatic activity or improved access to drug. We investigated the responses of the hepatocytes cultured in Si3N4-SC and collagen gel sandwich to APAP-induced hepatotoxicity, with hepatocytes cultured on non-sandwich collagen-coated coverslip surface as a control for drug accessibility. Hepatotoxicity by APAP, an analgesic, is mediated by the metabolism of APAP into a toxic intermediate, N-acetyl-p-
Fig. 5. Biliary excretory function of hepatocytes in the Si3N4-SC was established earlier than in the conventional collagen gel sandwich. Biliary excretory function was assessed by fluorescein diacetate (FDA) staining at day 1, day 2 and day 3 of culture. (A–C) FDA staining of hepatocytes in collagen gel sandwich; (D–F) FDA staining of hepatocytes in Si3N4-SC. The ratio of excreted (inter-cellular) FDA to intra-cellular FDA is indicated on the lower right corner of each image. (Data are mean ± s.e.m. of 10 images from two independent experiments.)

Fig. 6. Hepatocytes in the Si3N4-SC reestablish polarity earlier than conventional collagen gel sandwich. Hepatocyte polarity was indicated by double immunostaining of bile canalicular transporter, multidrug resistance protein 2 (Mrp2) (green) and basolateral marker CD147 (red), at day 2, day 4 and day 6 of culture. (A–C) Mrp2 and CD147 double immunostaining of hepatocytes in collagen gel sandwich; (D–F) Mrp2 and CD147 double immunostaining of hepatocytes in Si3N4-SC. Mrp2 located in the apical domain was indicated by arrows and quantified by image processing. The ratio of Mrp2 signal to CD147 signal is placed on the lower right corner of each image. (Data are mean ± s.e.m. of 10 images from two independent experiments.)
benzoquinoneimine (NAPQI), by CYP450 enzymes [42,43]. After exposure to 10 mM and 40 mM of APAP for 6 h, hepatocytes’ viability was monitored by MTS assay (Fig. 8). At 10 mM APAP, cell viability in the Si3N4-SC was 34%, compared to 63% in the collagen gel sandwich and 92% on collagen-coated coverslip surface. Exposure to 40 mM of APAP resulted in a viability of 20% in the Si3N4-SC as compared to 45% in the collagen gel sandwich and 58% on collagen-coated coverslip surface. These results indicate that hepatocytes in the Si3N4-SC were about twice as sensitive to APAP-mediated hepatotoxicity as compared to hepatocytes in the collagen gel sandwich and three times as sensitive as that on the collagen-coated coverslips.

4. Discussion

The microfabricated Si3N4 membrane based sandwich culture (Si3N4-SC) was developed to achieve improved and configurable mass transport as compared with a conventional collagen gel sandwich culture. Improved mass transport in the Si3N4-SC was achieved by using microfabricated, ultra-thin porous Si3N4 membrane for the top support of the sandwich culture and replacing the collagen gel with galactose ligands. Diffusion studies of FITC-dextran through three different sandwich culture configurations (Fig. 3) verified that using the Si3N4 membranes with galactose ligands exhibited improved mass transport properties over a conventional sandwich configuration. There are two possible reasons for the improved mass transport. Firstly, the galactose ligands immobilized porous Si3N4 membrane has thickness of 1.5 μm while the collagen gel coated PC membrane is 100–200 μm thick. Secondly, the pores in the Si3N4 membranes have direct access to the cells, while the pores in the PC membranes are blocked by the collagen gel which might act as a transport barrier for molecules. Besides improving the mass transport over conventional collagen gel sandwich, mass transport of the Si3N4-SC can be configured by...
changing the porosity of the Si3N4 membranes by either changing the pore size or inter-pore distance, obtaining an optimal configuration for a hepatocytes culture. In our study, we changed the porosity of the Si3N4 membranes by varying the inter-pore distance for a 20 μm pore.

To replace collagen, galactose was used since it interacts with the asialoglycoprotein receptors (ASGPRs) on hepatocytes to maintain their differentiated functions [23,24]. While galactose-immobilized substrates have been used for hepatocyte culture [23,24], the morphology of the hepatocytes in the galactose-immobilized Si3N4-SC was distinctly different when compared with that of monolayer culture on galactose-immobilized substrates (Fig. 4). Hepatocytes cultured on galactose-immobilized substrates readily form three-dimensional (3D) spheroids, which eventually detach from the substrates (Fig. 4A–F) due to the relatively weak interaction between galactose and ASGPRs [23,24]. In comparison, hepatocytes in the Si3N4-SC exhibited a stable morphology throughout the 6 days of culture (Fig. 4G–I). The stabilization of hepatocyte morphology in the Si3N4-SC may be attributed to the presence of the top layer of the Si3N4-SC (i.e., the Si3N4 membrane) to physically restrict the aggregation of the hepatocytes into 3D spheroids. Biochemical interactions exerted by the galactose ligands at both sides of hepatocytes monolayer may also counteract the strong cell–cell interaction required for spheroid formation.

Compared with hepatocytes cultured in the conventional collagen sandwich configuration, the Si3N4-SC showed improved mass transport (Fig. 3) and the cultured hepatocytes showed better ammonia removal (Fig. 7A), earlier repolarization (Figs. 5 and 6), higher P450 enzymatic activity (Fig. 7C) and an increase in drug sensitivity (Fig. 8). The increased drug sensitivity is likely due to a higher metabolic activity of the CYP450 enzymes since drug access to hepatocytes in the negative control (collagen-coated coverslip surface) would be better than that in the Si3N4-SC. The microfabricated porous Si3N4 membrane allows the mass transport to be configured systematically for functional optimization, by changing the pore size and inter-pore distance of the Si3N4 membrane over a very wide range, beyond what is achievable with the commercially available track-etched PC membranes. Thus the Si3N4-SC can serve as a reproducible and reliable drug testing platform for hepatotoxicity.

5. Conclusions

We have developed a sandwich culture configuration for primary hepatocytes based on ultra-thin, porous Si3N4 membranes with well-defined pore size and inter-pore distance and immobilized galactose ligands to maintain hepatocyte differentiated functions in vitro. Primary rat hepatocytes cultured in this Si3N4-SC showed stabilized morphology, earlier repolarization, high levels of differentiated functions and higher sensitivity to APAP-induced hepatotoxicity than those cultured in conventional collagen gel sandwich. Thus, the Si3N4-SC has all the essential cell-supporting and mass transport properties to serve as a promising platform for drug testing.

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