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Engineering of polarized tubular structures in a microfluidic device to study calcium phosphate stone formation†

Zengjiang Wei^{a,b}, Prince K. Amponsah^c, Mariyam Al-Shatti^c, Zhihong Nie^b, and Bidhan C. Bandyopadhyay^c

Zhihong Nie: znjie@umd.edu; Bidhan C. Bandyopadhyay: bidhan.bandyopadhyay@va.gov

^aResearch Institute of Materials Science, South China University of Technology, Guangzhou, 510640, China

^bDepartment of Chemistry and Biochemistry, University of Maryland College Park, MD, 20742, USA

^cCalcium Signaling Laboratory, DVA Medical Center, 50 Irving Street NW, Washington, DC, 20422, USA

Abstract

This communication describes the formation of tubular structures with a circular cross-section by growing epithelial cells in a microfluidic (MF) device. Here we show for the first time that it is possible to form a monolayer of polarized cells, embedded within the MF device which can function as an *in vivo* epithelia. We showed: i) the overexpression of specific protein(s) of interest (*i.e.*, ion channel and transport proteins) is feasible inside tubular structures in MFs; ii) the functional kinetic information of Ca^{2+} in cells can be measured by microfluorimetry using cell permeable Ca^{2+} probe under confocal microscope; and iii) calcium phosphate stones can be produced in real time in MFs with Ca^{2+} transporting epithelia. These data suggest that tubular structures inside this MF platform can be used as a suitable model to understand the molecular and pharmacological basis of calcium phosphate stone formation in the epithelial or other similar cellular micro environments.

Introduction

Tubular structures, such as renal tubules, salivary ducts and other similar epithelia, operate important mechanical functions. They not only enable the quick movement of materials (*i.e.*, nutrition, oxygen, waste, *etc.*) throughout the organism, but also prevent the formation of deposits or blockage.¹ These deposits that reduce the dynamic flow of fluids are often caused by the biomineralization of calcium phosphate. Such extra-skeletal calcium deposits are often generated in hollow tubes with a combination of interactions between the fluid flow and the tubular structure,^{2–4} suggesting a common mechanism for biomineralization of calcium phosphate within the above tubular structures.⁵ However, elucidation of the mechanism of such deposition of calcium phosphate has not yet been demonstrated due to the lack of *in vitro* models of the structural replica. Despite extensive research being conducted over the past century, the mechanism by which crystals nucleate, grow, and aggregate into stones is still poorly understood.^{4,5} These biologically controlled mineralization processes inside the human body usually take place within 3D tubular

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Correspondence to: Zhihong Nie, znjie@umd.edu; Bidhan C. Bandyopadhyay, bidhan.bandyopadhyay@va.gov.

structures that are actively and precisely regulated by the surrounding microenvironment.^{2,6} Moreover, both the compositions of renal fluids and the biological properties of lumen walls (*i.e.*, the activity of the channels and the transporters) play a critical role in such processes.⁷ Previous studies regarding the mechanisms of stone formation have been conducted by either drawing conclusions indirectly *via* characterizing the morphology and composition of extracted stones^{2,8} or by growing the stone-forming crystals in bulk systems (*e.g.*, petri dishes).⁹ These approaches lack both the real kinetic information and the dynamic control over the microenvironment, which are critical for understanding the process of stone formation in human tubules.

Microfluidic (MF) technology has shown great potential in the study of biomedicines,^{10,11} tissue engineering,¹² and cellular biology.^{13–17} Compared to conventional techniques, MF-based cell culture offers advantages such as low consumption of reagents,¹⁸ highly parallel or automatic processing,^{19,20} better mimicry of *in vivo* cellular microenvironment,^{21,22} and precise control over fluids or chemical delivery.^{15,23,24} Recently, Suh *et al.*²¹ showed that MFs provides fluidic environments for the culture of renal tubular cells on the planar substrate within microchannels. However, the planar monolayer of cells is significantly different from 3D tubular structures of *in vivo* systems. Microchannels have also been used as cylindrical cell scaffolds for seeding endothelial cells and creating artificial blood vessels *in vitro*.²² For example, Kumacheva *et al.*²² cultured endothelial cells over the wall of microchannel to mimic a small diameter blood vessel. To date, cellular lumens, however, have never been developed to study the stone formation *in situ*. This is largely due to the lack of optimal design of MF platform with the formation of polarized tubular structures. This communication reports the successful culture of two different model tubular cells—salivary ductal cells and renal proximal tubular cells—to form polarized monolayers of cells within MF channels. We further demonstrated the preliminary study of the mechanism of calcium phosphate deposition in 3D human tubular structures created within MFs. The unique MF platform enables us to precisely regulate the spatiotemporal composition of fluids, hydrodynamic flow, and chemical stimuli in the 3D biological tubules integrated inside the microchannels. In this model system, we monitored the deposition of calcium phosphate *in situ* inside a 3D tubular structure under optical microscope and confocal Raman microscope. This platform enables us to mimic the biological process of stone formation, and to address critical factors in this process. Our study offers new scenarios of opportunities in the areas including implantable organs or devices,²⁵ drug and toxic screening,²⁶ and cell biology,²⁷ by reconstructing tubular tissues *in vitro*.

Results and discussion

Culture of ion-transporting epithelial cells in MF devices

To test the feasibility of creating 3D tubular structures with a single layer of cells in a MF environment, we first used submandibular immortalized epithelial cells (SMIE; a gift from Dr Bruce J. Baum, NIDCR) as a cellular component. These salivary ductal cells²⁸ were cultured on the surface of microchannels, and grew and differentiated till uniformly covered over all the inner channel of MF devices under the continuous flow of nutrition and carbon dioxide. Fig. 1A shows a representative MF device with an inner diameter of ~400 μm of the microchannel (see Figs SI1 and SI2[†] for more details). Unlike the commonly used rectangular channels, the circular cross-section of our microchannels enables the uniform seeding of cells all over their walls.²¹ To facilitate the adhesion of cells, the surface of microchannel was coated with a thin layer of glass, followed by the absorption of

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fibronectin.²⁹ The SMIE cells were injected and seeded over the wall of the microchannel which supports the production of tubular shapes of the monolayer of lining cells. The DIC micrograph in Fig. 1B shows the growth of the cells inside the microchannel. The uniformity of distribution of cells grown over the microchannel was further confirmed by staining those cells using two nuclear stains, propidium iodide (PI) and 4',6-diamidino-2-phenylindol (DAPI). Fig. 1C,D shows a pattern of the distribution of cellular structure and the high-affinity regions for these fluorochromes. Images here also represent the overall viability of the cells cultured in the microchannels.

Characteristics of cells in MF devices

For the past several years, we have been working on understanding the mechanism of calcium biomineralization in pathological conditions.^{29,30} The MF platform we developed allows the integration of all necessary components in a single tubular structure for exploring such biomineralization process. To achieve full function of tubular structures, the engineered polarized epithelia must possess an asymmetric distribution of various ion channels and transporters, and a facilitated water-permeability pathway, all in the plasma membrane. Hence, we first tested whether salivary gland ductal cell line is in fact capable of attaining a polarized monolayer organization inside the tubular structure. We show here for the first time that the cells grown in the MF channel represent as a monolayer structure that expressed a key plasma membrane protein, Na^+ , K^+ -ATPase (Fig. 2A). This integral membrane protein distributes in a restricted domain (basolateral) of the plasma membrane in a polarized manner. This protein not only acts as an enzyme, but also creates ionic gradients that drive the net movement of glucose, amino acids, and ions across the entire epithelium. Sodium transport across epithelia is important, not only because of the physiological role of this ion, but also because the Na^+ pumped out of the cytoplasm by Na^+ , K^+ -ATPase creates an asymmetry between cytoplasmic Na^+ and extracellular Na^+ . This asymmetry establishes an electrochemical gradient across the plasma membrane that provides the driving force and increases the substrate affinity for a wide variety of Na^+ /glucose, Na^+ /amino acid, Na^+ / K^+ , and Na^+ / Ca^{2+} co-transporters (usually, carriers only acquire an affinity for the co-transported substance once they combine with Na^+).³¹ Not surprisingly, Na^+ transport is closely modulated in response to a multitude of physiological, pharmacological, and pathological conditions. In epithelial cells, tight junctions mark the presence of such asymmetry and separate the plasma membrane into distinct apical and basolateral domains, as well as provide a barrier function, limiting the paracellular movement of water, proteins and small solutes.³² We show here the expression of a key tight junction protein, Zonula occludens (ZO-1), at the apico-basal junction (Fig. 2B) when grown on a MF channel support. These data suggest that the polarization of the epithelial cells makes it possible to test the dynamic process of calcium phosphate biomineralization *in vitro* in a cellular platform.

Overexpression of green fluorescence protein (GFP) in the 3D tubular structures inside MFs

The formation of this cellular tubule in MFs is applicable to other cell types. For example, the culture of renal tubular cells produced well-defined renal tubules within MFs. This offers an excellent platform to study the dynamic process of stone formation. Fig. 3A shows expression of GFP as a model protein in the human proximal tubular (PT) cell line (HK2; ATCC, Manassas VA) cells grown in MF channel. Such an expression demonstrates the possibility of introducing any transporter/ion channel within the MF channel to test the role of these proteins in the process of stone formation. To demonstrate that these cells are distributed uniformly in a monolayer of lining PT cells, we have tested the location of epithelial basolateral marker protein. Fig. 3B represents a cross-section of MF channel with HK2 and the distribution of key plasma membrane protein, Na^+ , K^+ -ATPase basolaterally

inside the proximal tubular cells. The fluorescence distribution along the longitudinal cross-section of the tubules indicates the typical basolateral staining in the monolayer of cells on the wall of the microchannels.

Functional kinetic information of Ca^{2+} in the cells using cell permeable Ca^{2+} probe fluo-4 under confocal microscope

The nucleation of calcium phosphate stone depends on the elevation of the concentration of calcium ions ($[\text{Ca}^{2+}]$) beyond physiological range in the tubular lumen. We, therefore, investigated how the changes in luminal $[\text{Ca}^{2+}]$ affect the cellular dynamics of intracellular $[\text{Ca}^{2+}]$. Our results showed that the intracellular $[\text{Ca}^{2+}]$ profile increased with the addition of luminal Ca^{2+} in a dose dependent manner (up to 5 mM) (Fig. 4A, B). Furthermore application of Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA) abolished this response (Fig. 4C). Interestingly, the application of another polyvalent cation gadolinium (Gd^{3+}) also increased the intracellular $[\text{Ca}^{2+}]$. This observation suggest that the presence of other ions in the tubular lumen may also significantly influence the uptake of Ca^{2+} by cells (see Fig. SI3[†]).

Calcium phosphate stone formation the 3D tubular structures

Kidney stones are primarily (>50%) composed of calcium oxalate stones, while calcium phosphate stones are less common. However, there has been reported a rapid increase in the prevalence of calcium phosphate stones in the past two decades.³³ Such stones are generally formed in the Loops of Henley, which are located just before the proximal tubule. Many risk factors have been documented for developing such stones *in vivo*. Perhaps two most critical factors for calcium stone formation are the excessive urinary calcium excretion (so-called “hypercalciuria”) and high urine pH.⁵ However, the exact mechanism for the formation of calcium phosphate stones is still unclear.³⁴ The 3D cellular tubules we generated within MFs enabled us to simulate an *in vivo*-like renal microenvironment for the real time study of the mechanism of calcium phosphate kidney stone formation. As a preliminary demonstration, we showed the *in situ* deposition of calcium phosphate stone inside the proximal renal tubules. The solutions of CaCl_2 and Na_3PO_4 in Hank’s Buffered Salt Solution (HBSS; pH 7.4) were introduced side by side from two inlets of the microchannel, and mixed rapidly inside the renal tubules. After about 10 mins, crystals of calcium phosphate started initiating and depositing on the cellular walls (black clusters in Fig. 5A, B). The size of crystals evolved with time and reached up to several micrometers. The formation of calcium phosphate deposits can be monitored *in situ* by Confocal Raman microscopy. As shown in Fig. 5C, a new peak of near 960 cm^{-1} (corresponding to the ν_1 phosphate stretching) appeared, suggesting the presence of hydroxyapatite calcium phosphate deposits, about 15 mins after two solutions were flew inside the tubules.³⁵ Other types of calcium stones (*i.e.*, calcium oxalate stones, and CaHPO_4 stones), which count for large percentages of kidney stones, can be also deposited on the surface of cell walls (see Figs SI4 and SI5[†]). The appearance of the calcium stones can be directly visualized under optical microscope. For example, under optical microscope, we observed that calcium oxalate crystal nucleated on the surface of cell walls, and quickly propagated to form clusters of crystals in about 20 mins (see Fig. SI5[†]). These preliminary results clearly indicated that the cellular structures within microchannels offer us a novel platform for real time observation of the kinetics of kidney stone formation. A more detailed study of the mechanism of calcium phosphate stone formation in this *in vivo*-like tubular environment is currently ongoing in our laboratory, and the results will be reported in a near future.

Conclusion

In conclusion, we present here the creation of biological lumens with circular shapes within MF channels, and the implementation of such tubular structures in the study of biologically controlled calcium phosphate stone formation. For the first time, we showed that cells (*i.e.*, renal/salivary tubular cells) grown in microchannels generated functional polarized monolayer as indicated by immunofluorescence analysis of the key tight junction proteins and epithelial markers. This strategy can be generalized to culture and form other types of cellular tubular structures in the MFs. In contrast to planar cell layer, the 3D circular shaped lumens recreate a *in vivo*-like environment for addressing important biological questions. We further demonstrated the *in situ* deposition of calcium phosphate stones on the inner surface of proximal renal tubules, suggesting that the cellular tubular structures offer an *in vivo*-like environment for the real-time observation of kidney stone formation. This work constitutes an important step towards i) the recreation of nephron lumens for the *in vitro* study of kidney-related diseases such as kidney stone formation, and ii) a new platform for cell-based rejuvenation therapy and screening of drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (A). A photograph of a MF device with circular cross-section. Inset shows the cross-section of the channel. (B–D). Monolayer of salivary ductal cells grown in 3D MFs: Differential interference contrast microscopy (DIC) micrograph (B), PI stained nucleus (C), and DAPI stained nucleus (D). Scale bars are 200 μm in (C) and (D).

Fig. 2. (A, B). Representative confocal images of polarized salivary ductal cells grown in 3D MFs, showing (A). Na⁺, K⁺, ATPase (basolateral marker) and (B). ZO1 (tight junction marker). Scale bars are 200 µm.

Fig. 3.
A. Renal proximal tubules generated in 3D MFs (GFP expression). B. Na⁺, K⁺, ATP-ase staining HK2 proximal tubular cells. Scale bars are 200 μm .

Fig. 4. Intracellular Ca^{2+} profile of renal proximal tubular cells upon the introduction of (A) 1 mM Ca^{2+} (basal), (B) 5 mM Ca^{2+} , and (C) 5 mM EGTA, into the proximal tubules inside MFs. Scale bars are 100 μm .

Fig. 5.

(A, B). Optical microscope image showing calcium phosphate crystals deposited on the luminal side of the cells within MF channels at different focus *z*-planes: (A) focused on cell layer, and (B) focused on crystal layer. The black clusters are calcium phosphate deposits. (C). Raman spectra of bonded (in calcium phosphate precipitates; black curve) and unbounded PO_4^{3-} (in solution as ions; red curve), showing the feasibility of monitoring stone formation in MFs. The scale bars are 200 μm.