Evaluation of membrane filtration system using The "Pore Diffusion" for eliminating viruses

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ABSTRACT.   Here, we report a first study of virus removal by a novel membrane filtration system, named the "Pore Diffusion". The "Pore Diffusion" manipulated the direction of circulating flow from vertical to parallel to the membrane, thereby achieved to alter the trans-membrane pressure as low as possible. We compared the viral activity between before and after filtration by both infectivity assay and real-time reverse transcription-PCR. Among 4 "Pore Diffusion" modules tested, the big module with average pore size of 80 nm showed the highest log reduction value of viral activity. Our study shows the possibility of "The Pore Diffusion" to filtrate viruses from bioproducts without increasing the trans-membrane pressure, so that the filtration process can be carried out effectively and economically.
KEY WORDS: brownian motion, laminar flow, The Pore Diffusion, trans-membrane pressure, turbulence flow

To date, various products had been manufactured from biological origins, especially in field of pharmaceutical, cosmetic and food-related areas. As those biomaterials often contain various microorganisms, it is important to show that no infectious microorganisms had been contaminated. In particular, manuals regarding to the pharmaceutical products are tightly regulated to prevent microbial contamination. To do so, laws enforce manufacturers to make sure that there are no contamination from infectious bacteria as well as viruses [1]. However, as there are many viruses which are yet to be identified, it is impossible to detect contamination from such unknown viruses. Thus, pharmaceutical manufacturers often inactivate viruses comprehensively by heat/biochemical mean and membrane filtration.

On the other hand, no regulations with regard to viral contamination had been established for cosmetics and food-related areas, although there are regulations for bacterial contamination [7]. Usually, heat-inactivation is a common method used to remove bacterial contamination. However, there are various drawbacks with regard to heat inactivation. For example: 1) there is a trade-off between the effect of heat sterilization and physiological activity of protein by denaturation, 2) some spores are heat-resistant, and 3) heat-resistant toxins cannot be inactivated. It is commonly accepted that establishing heat-inactivation that can overcome these drawbacks is backbreaking. To solve these problems,

combination with other removal methods are desired, such as membrane filtration.

Membrane filtration is also a common method to remove microorganisms from biomaterials. In membrane filtration, trans-membrane pressure (TMP) is the major contributing factor for mobilization of materials. In theory, pores in filter capture microorganisms larger than their diameters, thereby "filtrates" the target microorganisms. This is called molecular sieve effect, and pore sizes are designed depending on what microorganism filters are designed to capture. Such filters against large microorganisms, such as bacteria, are already on market, and sometimes they had also been used as virus filters [4]. The simplicity of membrane filtration allows it as major option for filtration of various targets. However, clogging causes considerable problem in membrane filtration. The conventional membrane filtration requires relatively high TMP difference (>0.1 megapascal [Mpa]), which eventually clogs the membrane with crudes captured in pores. Thus, lowering the formation of clogs is desired to improve the efficiency of membrane filtration.

Notwithstanding the formation of clogs, it is true that membrane filtration can overcome the problems observed in heat-inactivation mentioned earlier [3]. Novel membrane filtration system is desired to provide reliable products without harmful substances. The Pore Diffusion is a novel membrane filtration system developed in Sepasigma Inc. (Kitakyushu, Japan), in which its idea was originally constructed by Kamide and Manabe to overcome the problems in conventional membrane filtration [2, 5]. Although the Pore Diffusion had already utilized for bacteria filtration, the ability for virus filtration is yet to be evaluated. The aim of this study is to validate whether the Pore Diffusion can remove viruses as well.

The basis of the Pore Diffusion with comparison to conventional membrane filtration system is illustrated

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in Fig. 1. The Pore Diffusion can utilize membranes with ranges of pore sizes from ultrafiltration (average pore size: 2–100 nm) to microfiltration (average pore size: >100 nm) level. The concept of the Pore Diffusion is to decrease TMP as low as possible. Lowered TMP improves filtration efficiency by improvement of molecular sieve effect, which often dysfunctions in conventional membrane filtration. Therefore, it is expected to overcome the problem of clogging when TMP is lowered.

The validation of filtration efficiency of the Pore Diffusion system had been done previously in 2012, upon commercialization of extracts of porcine-derived placenta as cosmetics. While processing this product, module named SepaΣpro (average diameter: 500 nm) was used to examine the filtration efficiency of bacteria in porcine-derived placenta. The previous study showed that the filtrated sample contained bacteria below the detectable level, without lowering the cytokine activity level of placenta itself [6]. The log reduction value (LRV) from this bacterial filtration was above 6, showing prompt potential of the Pore Diffusion system as filtration system. However, no study had been done since then to validate the filtration efficiency of the Pore Diffusion system for viral particles.

There are three foci of this study: 1) to evaluate the viral filtration efficiency of module with the Pore Diffusion systems designed in 2012, 2) to determine the pore diameter of the modules to achieve LRV >4 in subject virus, feline leukemia virus subgroup B (FeLV-B) and 3) to suggest the basic design of module with the Pore Diffusion system for filtration of both bacteria and virus, which will be applicable to refine the pharmaceutical, cosmetic and food-derived products. Overall, this study validates the possibility for developing a membrane filtration mechanism with the Pore Diffusion.

TE671 cells (human rhabdomyosarcoma) [12] and TEL-CeB6/pFBFeLV-B cells [8] were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO_2 in air using CO_2 incubators.

LacZ pseudotype virus of FeLV-B, termed *LacZ* (FeLV-B) (approximate diameter: 100 nm), was used for assaying the filtration efficiency. *LacZ* (FeLV-B) was obtained from TEL-CeB6/pFBFeLV-B cells [8]. Culture supernatants of the cells were collected and then filtrated with 800 nm membrane filter (Acrodisc; PALL Co., Ann Arbor, MI, U.S.A.). Filtered viruses were kept at −80°C until used.

Four types of modules were used in this study: Big Module with 500 nm or 80 nm pore, and Small Module with 500 nm or 80 nm pore. The reason why these four modules were chosen for filtration was mainly by the following two reasons: 1, to evaluate the pore size used to remove bacteria (500 nm) for virus filtration, and; 2, the importance of module size must be studied to apply in industrial scale filtration. Big Module which contains 0.2 m^2 of effective filtration area, and Small Module which contains 0.0057 m^2 of effective filtration area. The modules were supplied from SepaSigma Co., Ltd., with-
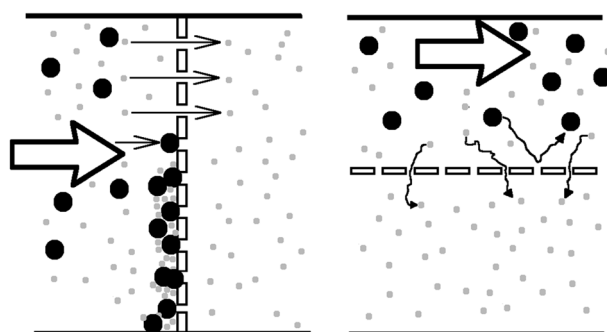

Fig. 1. Comparison of filtration basis between conventional (left) system and The Pore Diffusion system (right). Large black dots: adulterants. Gray small dots: water molecules. Large white arrow: the flow direction. Small black arrow: movement of the particles. In conventional filtration system, the TMP generated by the flow will clog the membrane with adulterant larger than designed pore size. The Pore Diffusion, on the other hand, is designed to avoid clogging by lowering the TMP by manipulating flow direction in parallel with membrane, so that only the Brownian motion will be used to filtrate samples.

out any modification from the module commercially available. Two types of flat sheet membrane (average pore sizes: 500 nm and 80 nm, respectively) were prepared from the cellulose acetate/acetone solution through the micro-phase separation method. The regenerated cellulose multilayer structure flat sheet membranes were prepared by treating cellulose acetate membrane with 0.1N NaOH for saponification reaction. The mean pore size was evaluated by the filtration rate method of pure water. The bubble point method was employed to confirm that there are no pores larger than 1 μm in the membrane. The pressure hold method was applied to test the integrity of modules before the filtration. Likewise, the same test was employed after the filtration to confirm the integrity of modules. Any data obtained from modules with insufficient integrity were discarded. There were two types of flow lines utilized in this filtration; the first line where the solution filters through the membrane, and: the second line where the solution circulates without filtering membrane. In Small Module, the tubing pump was connected to entry/exit sites in both flow lines. In Big Module, the magnet pump was connected to entry/exit sites in both flow lines. Seventy percent ethanol was circulated within the module for 15 min, subsequently by distilled water for 15 min. After distilled water circulated was drained from the module, viral solution was circulated until enough amounts were collected. In Big Module, the viral solution was suspended for approximately fifty-times in DMEM to increase the volume of total solution. Some of virus solution before the filtration were collected and labeled as "Before". Filtered viral samples were collected in autoclaved vessels and labeled as "After". The circulating solutions were collected in sterilized tubes and labeled as "Remained".

To titrate *LacZ* (FeLV-B) virus, LacZ marker rescue (LMR) assay was employed as described previously [9]. Briefly, three different viral solutions were prepared as men-

tioned earlier to compare the infectivity of *LacZ* (FeLV-B) before and after filtration. 1.6×10^5 of TE671 cells were plated on each well of 24-well plate (Thermo Scientific, Waltham, MA, U.S.A.) one day before inoculation. On the next day, each viral supernatant was serially diluted and immediately inoculated into TE671 cells under the presence of polybrene® (AL-177, Sigma-Aldrich, St. Louis, MO, U.S.A.) ($8\mu g/ml$), and incubated for 4 hr at 37°C with 5% CO_2 in air using a CO_2 incubator for viral adsorption. After viral adsorption, the inocula were replaced with 0.5 m*l* of fresh DMEM supplemented with 10% FCS and antibiotics, and further incubated for additional two days. Cells were then fixed with 1% glutaldehyde and stained with 1 mg/m*l* β-gal, and then, *lacZ*-positive foci were counted to titrate the infectivity as described previously [10].

Figure 2 shows how the filtration had impact on infectivity, using LMR assay. Big Module with 500 nm pore and Small Module with 80 nm also removed viruses at a certain extent, but the Small Module with 500 nm pore showed almost no effect to reduce infectivity by filtration. Table 1 summarizes LRV of 8 different experiments which show changes of *LacZ* (FeLV-B) infectivity before and after filtration. Among these we tested, the Big Module with 80 nm pore showed the most significant reduction of viral particle by filtration.

The viral RNA (*LacZ* gene) copy numbers were measured using real-time reverse transcription-polymerase chain reaction (RT-PCR) from samples tested for infectivity, to allow comparison between infectivity and actual RNA copy numbers in viral solution. Samples were treated with recombinant DNase I (Takara, Otsu, Japan) and recombinant RNase inhibitor (Takara) to eliminate free DNA, and then incubated in 37°C for 60 min and 75°C for 10 min to extract viral RNA. The viral RNA (*LacZ* gene) copy numbers were measured using One Step SYBR® PrimeScript™ PLUS RT-PCR Kit (Takara) according to the manufacturer's instruction. The primers used were designed to detect the *LacZ* gene (5′-GCGTGGATGAAGACCAGC-3′ and 5′-CGAAGCC-GCCCTGTAAAC-3′). The results obtained show somewhat similar results with the infectivity assay, despite lower LRV in general (Fig. 3 and Table 1). This is most obvious in Big module with 500 nm pore, which shows high numbers of retained viral RNA in samples after filtration, although the infectivity assay shows high LRV (Figs. 2 and 3, Table 1). The filtration by Big Module with 80 nm pore showed almost no difference of LRV between infectivity assay and RNA copy numbers (Fig. 3). Because of low filtration efficiency observed in infectivity assay with Small Module with 500 nm pore, the real-time RT-PCR for this particular sample was omitted.

There were no significant differences in both infectivity and viral RNA copy numbers between samples before filtration and remaining solutions (Figs. 2 and 3). Therefore, the LRV shown in this study represents the effect of filtration process without significant virus inactivation, as well as virus adsorption to the modules during circulation.

In this study, FeLV particles can be removed with LRV of above 3. The LRV showed in infectivity assay is somewhat larger than those observed in real-time RT-PCR (Table 1).
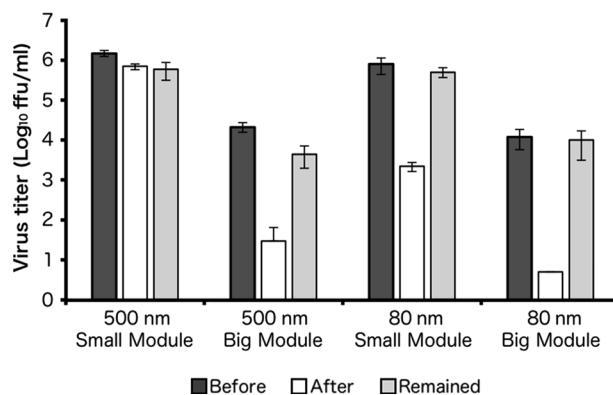

Fig. 2. Comparison of infectivity observed by LMR assay from solutions collected at: before/after filtration and remained circulating solution. Mainly, there are 4 different modules which had been tested for filtration efficiency of *LacZ* (FeLV-B): small module with 500 nm pore size, big module with 500 nm pore size, small module with 80 nm pore size and big module with 80 nm pore size. Filtered samples were serially diluted and inoculated into TE671 cells for LMR assay. The virus titer was expressed as f.f.u./m*l*.

Table 1. Filtration efficiency of the Pore Diffusion systems

Exp. No.	Pore size (nm)	Module size	Unit*	LRV**
1	500	Small	f.f.u.	0.33
2		Big		2.85
3		Small	RNA copy no.	NE***
4		Big		0.98
5	80	Small	f.f.u.	2.56
6		Big		3.38
7		Small	RNA copy no.	1.89
8		Big		3.20

*The filtration efficiency was calculated by subtracting the infectivity observed after the filtration from infectivity observed before the filtration using indicated unit in log scale. **LRV: Log reduction value; ***NE: not examined.

There could be two explanations for this phenomenon: 1) uninfectious virus particles were passed through the pores, and 2) PCR's detection limit. The first explanation is that there may be fractions of *LacZ* (FeLV-B) that passed through the filtration, but were not infectious so that it cannot be assayed by LMR assay. One possible explanation is the lack of envelope protein (Env) of the pseudotype virus prepared. *LacZ* (FeLV-B) was prepared from TELCeB6/pFBFeLV-B cells (TELCeB6 cells transduced with an expression plasmid of FeLV-B Env, termed pFBFeLV-B [8]). Presumably, some of the cells do not express FeLV Env sufficiently. Because TELCeB6 cells produce Env-less viral particles [9], TELCeB6/pFBFeLV-B would produce both Env-less defective particles and *LacZ* (FeLV-B) bearing FeLV-B Env. It is expectable that the Env-less virus particles will be reduced size in diameter. The Env-less virus particles are unable to infect the target cell and thereby cannot be titrated by the LMR assay. By contrast, Env-less virus particles virus will con-
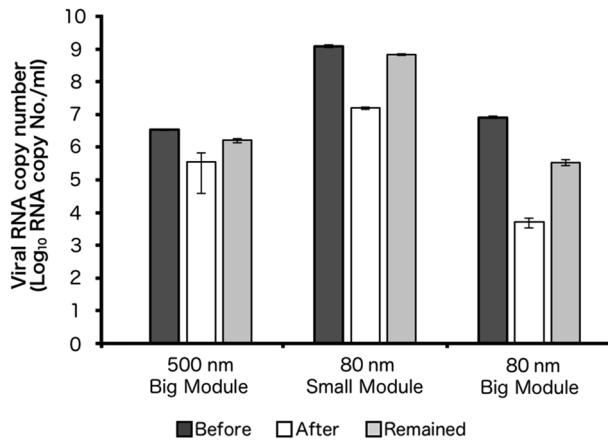
Fig. 3. Comparison of viral RNA copy numbers from solutions collected at: before/after filtration and remained circulating solution. There are 3 different modules which had been tested for filtration efficiency of *LacZ* (FeLV-B): big module with 500 nm pore size, small module with 80 nm pore size and big module with 80 nm pore size. Each sample was treated with DNase to eliminate free DNA and employed real-time RT-PCR to quantify the viral RNA copy numbers.
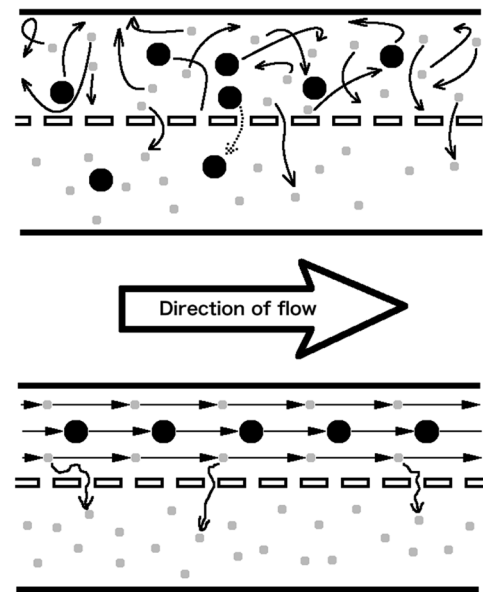

Fig. 4. Comparison between turbulent and laminar flow during filtration. The top panel shows the turbulent flow, and the bottom panel shows the laminar flow. In turbulent flow, the flow scatters the material inside the flow all over, resulting improper filtration. In laminar flow, on the other hand, larger substance will concentrate on center of flow, minimizing the encounter to the filtration membrane. This allows filtration to take place only by Brownian motion, and thereby, only small particles can pass through.

tribute to the viral RNA copy numbers in real-time RT-PCR, so the LRV will decrease because such inactive virus is also counted. The second reason is that PCR cannot precisely validate the RNA copy numbers if they are too low. This is because the negative control (diethylpyrocarbonate-treated water) used in this assay showed $1e^{-1}$ delta run at 35th cycle, whereas *LacZ* (FeLV-B) after filtration by Big Module with 80 nm pore showed 1 delta run at 34th cycle (data not shown). This indicates that RNA copy numbers present in filtered *LacZ* (FeLV-B) are almost equivalent to those in a negative control. However, the detection limit in this step is that this slight difference must be multiplied by 2×10^3 when converting from 0.5 microliter to milliliter scale. This enlarges the viral RNA copy numbers in samples after the filtration and therefore lowers LRV. Therefore, more validation is required to properly assess the value of RNA copy numbers obtained from samples removed by Big Modules.

As Table 1 shows, the viral filtration efficiency greatly increased when the module size was increased. The difference is even clearer in module with 500 nm pore, which showed roughly multiplied filtration efficiency four-fold in infectivity assay (but not in RNA copy numbers) when the module size was increased from "Small" to "Big". This phenomenon is interesting to note, because this observation contradicts with conventional filtration, which smaller the module size often results increased filtration efficiency. This is mainly because bigger the module size will provide larger space, and therefore is difficult to regulate the same filtration efficiency at every site of membrane [11]. The main reason why the opposite effect of size is observed in the Pore Diffusion is considered to be because of the state of flow. As illustrated in Fig. 4, circulating flow in small module forms the turbulent flow, whereas it will be laminar flow in big module.

The reason why such change occurs can be explained by the difference of module's design in each module. In the Small Module, the circulating flow directly hits the membrane (vertical flow), whereas in the Big Module, the circulating flow does not hit the membrane, and instead, it circulates module in parallel with membrane. This suggests that the Pore Diffusion works much better in laminar flow than turbulence, and it is desired to design the module which can use laminar flow. This also explains why Small Module with 500 nm pore showed significantly low LRV, because both pore size and module design were inappropriate. Therefore, this indicates the possibility that the module size can be maximized as much as possible without lowering the filtration efficiency, as long as the state of flow is kept at laminar flow.

Overall, our results answer questions we set in the introduction. To answer (1), this study showed that the Pore Diffusion system introduced in 2012 designed to remove bacteria (500 nm Big Module) can also remove viruses at a certain extent, but not good enough. In LMR assay, the filtration of FeLV-B in 500 nm Big Module shows the virus can be filtered for two orders of magnitude with this system. However, this system has not achieved the LRV of 6 which we originally set as a goal for filtration efficiency, and thus, we have to conclude that this module is not satisfactory for filtration of viruses sized 100 nm or less. Our results indicate that further improvement is necessary for this module to be used as the filter for virus removal. To answer (2), 80 nm Big Module was the better module with higher filtration

efficiency, although this system could not achieve the LRV of 4. Therefore, our results indicate that the pore size will need to be decreased further to remove viruses that are sized similar with FeLV-B (approximately 100 nm). Our expectation is that it will be about 50–60 nm (average pore size) to achieve LRV of>4 in FeLV-B. The pore size will need to be narrowed down further, if we are to remove smaller viruses. However, the narrowing down the pore size also has a side effect, which is the slowing down the productivity of filtration. This is even more important when filtrating virus from the placenta extracts, where the fluidity is denser than the virus solution we used. Therefore, the answer to (3) will be: the most important parameter for the design is to construct module which can remove with lowest TMP differences, as well as the state of flow to be laminar than the turbulent.

REFERENCES

1. Center for Biologics Evaluation and Research, Food and Drug Administration (FDA, U.S.A.). 1993. Points to consider in the characterization of cell line used to produce biological. pp.1–40, Silver Spring.
2. Kamide, K. and Manabe, S. 1981. Mechanism of permselectivity of porous polymeric membranes in ultrafiltration process. *Polym. J.* **13**: 459–479. [CrossRef]
3. Manabe, S. and Yamamoto, N. 1996. A novel virus removal filter and its application. *Biomedical Rev.* **6**: 95–109. [CrossRef]
4. Manabe, S. 2002. Novel application of virus removal membrane developed in order to minimize virus infection from Bio-drug. *Membrane* **27**: 2–8. [CrossRef]
5. Manabe, S. and Hanada, S. Separation module by plane membrane utilizing pore diffusion. JP Patent 4803341, August 19, 2011.
6. Manabe, S., Itai, T., Takeshita, S., Tanikawa, T. and Sawatani, K. 2012. Preparation method for essence extracted from pig placenta. *Placenta* **33**: P2.91, A124.
7. Mitsuse, K. 1995. A commentary on Japanese pharmacopoeia and microbiological examination of nonsterile products: microbial enumeration tests in Japan (in Japanese). *es* **1**: 1–4.
8. Nakata, R., Miyazawa, T., Shin, Y. S., Watanabe, R., Mikami, T. and Matsuura, Y. 2003. Reevaluation of host ranges of feline leukemia virus subgroups. *Microbes Infect.* **5**: 947–950. [Medline] [CrossRef]
9. Pizzato, M., Marlow, S. A., Blair, E. D. and Takeuchi, Y. 1999. Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction. *J. Virol.* **73**: 8599–8611. [Medline]
10. Sakaguchi, S., Okada, M., Shojima, T., Baba, K. and Miyazawa, T. 2008. Establishment of a LacZ marker rescue assay to detect infectious RD114 virus. *J. Vet. Med. Sci.* **70**: 785–790. [Medline] [CrossRef]
11. Sekiguchi, S., Ito, K., Kobayashi, M., Ikeda, H., Tsurumi, T., Ishikawa, G., Manabe, S., Satani, M. and Yamashiki, T. 1989. Possibility of hepatitis B virus (HBV) removal from human plasma using regenerated cellulose hollow fiber (BMM). *Membrane* **14**: 253–261. [CrossRef]
12. Stratton, M. R., Darling, J., Pilkington, G. J., Lantos, P. L., Reeves, B. R. and Cooper, C. S. 1989. Characterization of the human cell line TE671. *Carcinogenesis* **10**: 899–905. [Medline] [CrossRef]