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Résumé :

Le contexte de cette étude est la fabrication d'un substitut oesophagien par ingénierie tissulaire, à partir d'une matrice d'œsophage de porc décellularisé puis recellularisé avec des cellules humaines. Le bioréacteur choisi pour réaliser ces opérations est le RCCMax-dual (Synthecon), qui est adapté pour une géométrie tubulaire comme celle de l'œsophage. Ce dispositif permet de combiner un flux longitudinal du milieu circulant et un mouvement de rotation de l'ensemble du système de forme cylindrique. Les équations du mouvement sont résolues pour une particule qui se trouve donc à la fois entraînée par un écoulement de Poiseuille spiral et soumise aux forces de gravitation, d'Archimèdes, centrifuge, de Coriolis, et de frottement. Des conséquences pratiques sont déduites de cette analyse pour l'utilisation du RCCMax en ingénierie tissulaire, mais cette étude peut certainement être transposée à d'autres applications.

Abstract :

The context of this study is the development of a tissue-engineered esophageal substitute, constituted of an acellular matrix and seeded cells. Due to the tubular shape of the esophagus, use of a Rotary Cell Culture System (RCCMax- dual, Synthecon) as a bioreactor is appropriate, since it allows liquid flow within the esophagus, as well as a rotation in and around the tissue in two successive closed chambers. A mechanical characterization of this flow device is provided, showing that the suspending fluid exhibits some spiral Poiseuille flow and that, in a transverse section, a particle experiences simultaneously gravitational, Archimedes, centrifugal, Coriolis and drag forces. Motion equations are solved in order to determine the trajectories of the suspended particles or cells. Practical informations are deduced for tissue engineering applications with the RCCMax. This study may also be useful for other applications.

Keywords : rotating bioreactor ; spiral Poiseuille flow ; simulated microgravity ; tubular scaffolds ; tissue engineering

1. Introduction

Tissue engineering aims at constructing biological substitutes that can mimic native tissues. Engineered tissues may be used for diagnostic or research purposes ; they can also repair native diseased or injured organs. Some natural or biodegradable synthetic matrices, called scaffolds, are necessary to enhance cell adhesion, proliferation, and/or differentiation. Scaffolds may also be obtained from other human or animal tissues that are treated by chemical and mechanical means in

order to remove their own cellular components. 3-D dynamic culture systems, called bioreactors, allow to improve the oxygen and nutritional support to the cultured cells, and to generate controlled mechanical stresses.

In this paper, we study a mechanical device that presents an overall cylindrical geometry and combines a rotational motion around its longitudinal axis with continuous perfusion through the scaffold: the rotary cell culture system (RCCS), commercially available from Synthecon (Houston, TX) [1]. The idea of Rotating Wall Vessel (RWV) was initially proposed by NASA in the 1970's. The objective was to simulate microgravity conditions since the rotational motion can prevent sedimentation of the suspended particles. Several derivative systems are described in the literature, differing in vessel geometry (2 co-axial cylinders), aspect ratio (rotating discs instead of cylinders), and gas supply (with an internal semi-permeable membrane), and differing in their aims and applications also (cell culture, scaffold production, osteocytes' differentiation and bone matrix formation, 3-D tumor models, re-epithelialization of de-epithelialized tracheal scaffolds, …). A review of the existing devices that simulate microgravity and can be used for various tissue engineering applications may be found in [2]. Some industrial utility may probably also be found for such devices.

The group of CIC-IT-Bordeaux is involved in esophagus tissue engineering with the objective to create a human esophageal substitute and improve clinical results in diseased esophageal treatment and surgery (esophageal atresia, cancer, perforations, burns). Currently, the restoration of digestive continuity after esophagectomy is achieved through the interposition of a segment of the colon or by the tubulation of the stomach; however there are many post-operative complications such as anastomotic leaks, infections, etc. The development of a tissue-engineered esophageal substitute, constituted of an acellular matrix and seeded cells, is thus of great interest. Decellularized scaffolds are prepared from pig esophagus using mild detergents, acids, or enzymes to remove animal cells, in order to provide scaffolds for recellularization with human stem cells [3]. Due to the tubular shape of the esophagus, use of RCCS as a bioreactor is appropriate, since it allows liquid flow within the esophagus, as well as a mechanical rotation in and around the tissue in two successive closed chambers. The aim of this paper is to provide a mechanical characterization of this flow device, in order to determine: i) the velocity fields, pressures, shear stresses in the fluid without suspended cells, ii) the forces that act on a suspended cell and determine its motion.

2. Materials and methods

2.1 Description of the perfusion bioreactor and closed flow loop

A schematic representation of the RCCMax-dual esophagus bioreactor and of the flow bench is reported in **Figure 1**. The bioreactor consists of two successive cylinder chambers that rotate horizontally at the same constant angular speed. Some direct motor drive is used to rotate the cylinders. In each chamber, there are scaffold holders on which a tubular scaffold can be mounted and tied with a non-absorbable 2/0 USP suture. The scaffold thus rotates at the same angular velocity as the chamber wall. The chambers are connected to a media reservoir bottle, an oxygenator and a 4-rollers peristaltic pump (Watson Marlow 314D). One r.p.m. on the peristaltic pump provides a 0.5 ml/min flow rate through the tubing. Silicon tubing has a wall tube thickness 1.6 mm and internal diameter 1.6 mm. The oxygenator uses silicone membrane diffusion of gases. The reservoir is open to atmospheric pressure. The circulating medium contains Sodium Azide, Sodium Deoxycholate and DNaseI.

2.2 Analytical approach

2.2.1 Fluid motion

The fluid medium used for the decellularization experiments is considered as newtonian, with a viscosity η_f = 1 mPas and a density ρ_f = 1015kg/m^3. For the moment, the esophagus wall is assumed non-deformable and non-porous and its thickness (a few millimeters) is not taken into account. R_1 denotes the esophagus radius (R_1 = 6 mm) and R_2 the chamber radius (R_2 = 31.5 mm). Both cylinders (esophagus and chamber) are supposed "infinitely" long, with an axial symmetry.

In each chamber of the bioreactor, two distinct parts will be considered: **Part A** will refer to the perfusion inside the esophagus, and **Part B** will refer to the medium enclosed between the esophagus and the chamber wall.

In **Part B**, there is no fluid circulation (no longitudinal fluid velocity). The fluid rotates as a rigid body with an angular velocity ω throughout the domain (Couette flow). In classical cylindrical coordinates (O, r, θ, z), this would yield: no radial velocity and an azimuthal velocity U_θ equal to ω r, r being the radial coordinate ($R_1 < r < R_2$). In this environment, shear stresses are null.

In **Part A**, for a given value of the pump flow rate, Q, quantities of interest do not depend on time. The flow is driven by the combination of two factors: a constant axial pressure gradient ($-\frac{\partial p^*}{\partial z}$, along Oz) and the rotation of the dual chamber. Velocity continuity prevails at the wall, due to the no-slip boundary conditions. This results in an exact superposition of an axial parabolic velocity profile U_z (mean velocity U_{mean}) and an azimuthal solid-body rotation U_θ depending only on the radial coordinate r .

This type of flow is known as the rotating Hagen-Poiseuille flow, or spiral-Poiseuille flow. The pathlines are helix curves. The flow is characterized by two non-dimensional control parameters: the streamwise Reynolds number:

$$R_{ez} = \frac{\rho_f \, U_{mean} \, (2R_1)}{\eta_f} \qquad (1)$$

and the azimuthal (or rotational) Reynolds number:

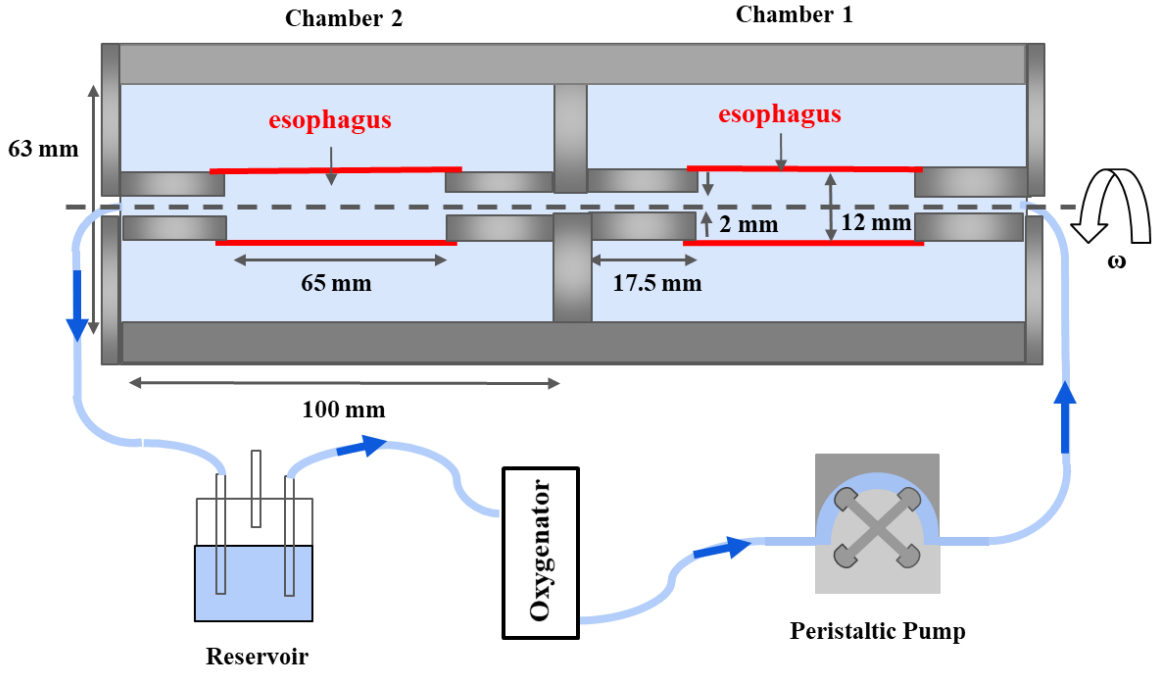

Figure 1 - Schematic representation of the experimental set-up.

$$R_{e\omega} = \frac{\rho_f (\omega R_1)(2R_1)}{\eta_f} \quad (2)$$

Tangential shear stresses in the azimuthal direction are null and tangential shear stresses in the axial direction may be calculated as:

$$\tau_{rz} = \eta_f \frac{\partial U_z}{\partial r} = -\frac{r}{2}\left(-\frac{\partial P^*}{\partial z}\right) \quad (3).$$

They are maximal at the wall of the esophagus ($r = R_1$).

It is important to mention that, both in **Part A** and **B**, the radial projection of Navier-Stokes equations can be simplified as:

$$\frac{\partial P^*}{\partial r} = \rho_f \frac{U_\theta^2}{r} = \rho_f \frac{\omega^2 r^2}{r} = \rho_f \omega^2 r \quad (4),$$

thus demonstrating that a positive radial pressure gradient exists in each domain.

2.2.2 Suspended particle motion

In order to be able to describe the motion of a particle relative to the rotating fluid, a rotating frame (O, x, y, z) is considered. Conversely, (O, X, Y, Z) is a ground-based frame (fixed). Since the esophagus cylinder and the chamber cylinder are concentric, the (OZ) and (Oz) axis are the same. Unit vectors associated with (OXY) are denoted $\mathbf{e_X}$ and $\mathbf{e_Y}$, and unit vectors associated with (Oxy) are denoted $\mathbf{e_x}$ and $\mathbf{e_y}$. The (Oz) and (OZ) unit vectors are denoted $\mathbf{e_z}$, so that the frames are direct. The gravitational acceleration **g** is perpendicular to the rotation axis (Oz); it is directed down the (OY) axis.

The frame (O, x, y, z) rotates counter-clockwise about the (Oz) axis with a constant angular velocity ω. The rotating vector is thus: $\mathbf{\Omega} = \omega\, \mathbf{e_z}$.

We consider a non-deformable spherical particle with radius a and density ρ_p (slightly higher than the fluid density ρ_f). The mass of the particle is thus:

$m_p = \rho_p V_p$, where V_p is the particle volume ($V_p = \frac{4}{3}\pi a^3$) .

Since the particle is positively buoyant, it experiences sedimentation while the chamber and the fluid are rotating. Introducing a buoyancy corrected mass, $m_b = (\rho_p - \rho_f) V_p$, the buoyancy corrected weight of the particle is: $m_b\, \mathbf{g}$, where we have to consider the projection of the gravitational acceleration **g** in the rotating frame:

$$\boldsymbol{g} = \begin{vmatrix} -g\sin(\omega t) \\ -g\cos(\omega t) \\ 0 \end{vmatrix} \quad (5).$$

Since the particle is small and the velocities are moderate, an appropriate estimation of the viscous drag may be obtained using Stokes approximation:

$\mathbf{D} = -k\,\mathbf{v}$, where the coefficient k is given by : $6\pi\,\eta_f\, a$, and **v** is the particle velocity.

Since the fluid is in solid body rotation, the pressure gradient acting on ($\rho_f V_p$) opposes the centripetal force on ($\rho_p V_p$), and the resulting force will be written as:

$$\begin{vmatrix} -m_b\,\omega^2 x \\ -m_b\,\omega^2\, y \\ 0 \end{vmatrix} \quad (6)$$

The particle is also submitted to the Coriolis acceleration:

$$2\,\mathbf{\Omega} \wedge \boldsymbol{v} = \begin{vmatrix} \mathbf{0} \\ \mathbf{0} \\ 2\,\omega \end{vmatrix} \wedge \begin{vmatrix} \dot{\boldsymbol{x}} \\ \dot{\boldsymbol{y}} \\ \dot{z} \end{vmatrix} = \begin{vmatrix} -2\,\omega\dot{y} \\ 2\,\omega\,\dot{x} \\ 0 \end{vmatrix} \quad (7)$$

Gathering all, the motion of the particle in the rotating frame , in **Part B**, is governed by the following differential equations:

$$m_p\,\ddot{x} = -k\dot{x} + m_b\omega^2 x + 2m_p\omega\dot{y} - m_b\,g\,sin\,(\omega t) \quad (8)$$

$$m_p\,\ddot{y} = -k\dot{y} + m_b\omega^2 y - 2m_p\omega\dot{x} - m_b\,g\,cos\,(\omega t) \quad (9)$$

Equations (8-9) indicate that particle motion may be affected by: density difference between fluid and particle, vessel rotation rate, fluid viscosity and particle radius.

In **Part A**, due to the fluid perfusion, a longitudinal motion of the particle exists. The equation of motion for the relative particle displacement is:

$$m_p\,\ddot{z} = -k\dot{z} \quad (10)$$

Additionally, the Poiseuille flow shear rate (radial variation of the longitudinal velocity) can induce a torque on the particle associated with a lift force and a radial migration. We have checked that this effect is negligible in the problem we study here.

3. Results

3.1Hydrodynamic calibrations

The effects of changes in operating conditions, including rotation rates and fluid perfusion rates, have been investigated. The chosen values were in agreement with the literature survey for this type of tissue engineering applications. An estimation of the flow entrance length in the esophagus is provided by the classical formula:

$$L_e \approx 0.05\,R_{ez}\,(2R_1) \quad (11).$$

Typically, for a flow rate Q = 25 ml/min, U_{mean} = 3, 68 mm/s, R_{ez} = 45, and L_e = 27mm. For ω = 15 r.p.m., the azimuthal Reynods number is $R_{e\omega}$ =115. The Reynolds numbers are thus sufficiently small for the flow to be considered laminar, and the entrance length not too important.

3.2 Order of magnitude of Coriolis force on the particle

Let us suppose that a cell can be represented by a spherical particle with diameter 2a = 15 microns and density ρ_p = 1070 kg/m^3 (the cell volume V_p will thus be 1767 μm^3 and its mass m_p = 1, 89 10^{-12} kg). We consider first the sedimentation equilibrium velocity, u_p, for such a particle suspended in a non-rotating fluid. In these conditions, the particle experiences drag and Archimedes forces against gravity. All these forces are directed along (OY) (vertical). The velocity u_p is given by the well-known Stokes formula:

$$u_p = \frac{2\,g\,(\rho_p - \rho_f)}{9}\,a^2\,\frac{1}{\eta_f} \quad (12)$$

With our numerical data, this yields: u_p = 6.74 μm/s, which is 3 orders of magnitude smaller than the fluid velocities.

Similarly, a Reynolds number based on the particle diameter 2a and terminal velocity u_p can be evaluated as:

$$R_e^p = \frac{\rho_f\;2a\;u_p}{\eta_f} \quad (13)$$

Its value is: $R_e^p = 0.0001$ (five or six orders of magnitude smaller than the fluid Reynolds).

Coming back to the rotating fluid and rotating frame (Oxyz), the sedimentation velocity u_p is the particle velocity relative to the rotating frame, and is thus involved in the evaluation of Coriolis acceleration, which norm can be expressed as: 2 ω u_p. For ω = 15 r.p.m., Coriolis acceleration scales

as: 2,12 10^{-5} m/s^2. This has to be compared to the centrifugal acceleration $\omega^2 r$. If the $\omega^2 r$ term is evaluated at a radial distance r = 3 mm (inside the esophagus), its value is 7,4 10^{-3} m/s^2; if it is evaluated at r = 2 cm (between the esophagus and the chamber wall) , its value is 49,3 10^{-3} m/s^2 . It may thus be concluded that the ratio of Coriolis acceleration to centrifugal acceleration is very small (of order 10^{-3}), and that Coriolis force may be neglected in Equ. (8-9).

3.3 Longitudinal motion of the particle inside the esophagus

Equation (10) indicate that inertial effects are balanced by the frictional force exerted on the sphere by the fluid. The mathematical solution for such an equation involves an exponential term decreasing with time: exp(-kt/m_p). Since m_p = 1, 89 10^{-12} kg, and k (defined as $6\pi\ \eta_f\ a$) equals 1.41 10^{-7} Pa.s.m, the ratio k/m_p = 7.48 10^4 s^{-1}. The exp(-kt/m_p) term is thus essentially transient and will decay very quickly. It can be ignored, and the absolute longitudinal velocity of the particle (in the laboratory frame) can be assumed to be roughly the same as the fluid velocity. This result is consistent with the fact that the particle radius is much smaller than the esophagus radius (a / R_1 = 7.5 10^{-6} m / 6 10^{-3} m ≈ 10^{-3}). The particle may be considered as a "tracer" and follows the fluid with the same speed as the local Poiseuille velocity. A key point for tissue engineering applications is the residence time of a suspended cell in the bioreactor. Based on U_{mean} velocity, this residence time can be evaluated as: Δt = esophagus length / U_{mean}. Since the esophagus length is roughly equal to 65 mm, for U_{mean} = 3,68 mm/s, a 17.7 s residence time is found.

3.4 Rotating motion of the particle

Multiplying Equ. (9) by the complex number i (i^2 = -1) and adding Equ. (8), this coupled system can be transformed in one equation in the complex domain as follows:

$$\ddot{s} + \left(\frac{k}{m_p} + 2\,i\,\omega\right)\dot{s} - \frac{m_b}{m_p}\omega^2 s = -\frac{m_b}{m_p}\,g\,i\,e^{-i\omega t} \quad , \quad \text{where } s = x + iy \qquad (14).$$

This equation is consistent with the work of Kessler et al. [4]. A rather simple solution of Equ. (14) may be obtained after dropping terms of smallest order of magnitude:

$$x(t) = \left(x_0 - \frac{u_p}{\omega}\right) e^{\frac{t}{\tau}} + \frac{u_p}{\omega}\cos(\omega t) \qquad (15) \qquad \text{and} \qquad y(t) = y_0\, e^{\frac{t}{\tau}} - \frac{u_p}{\omega}\sin(\omega t) \qquad (16),$$

where $\tau = k / m_b\ \omega^2$ has the physical meaning of a centrifugal time, and a numerical value of 5.9 10^5 s (obtained with k = 1, 41 10^{-7} Pa.s.m, ω = 1.57 s^{-1}, m_b = 9, 72 10^{-14} kg), and (x_0, y_0) denotes the initial position of the particle at t = 0. The particle follows a periodic nearly circular path in the clockwise direction, associated with a very slow centrifugal drift towards the esophagus wall.

Inside the esophagus (**Part A**), this nearly circular motion is combined with the longitudinal motion. This generates spiral trajectories. Equ. (15) and (16) show that the centrifugal drift of the particle is governed by the centrifugal time, τ, and that the radius of the orbit is proportional to the sedimentation velocity u_p and inversely proportional to the rotation rate ω. The centrifugal shift is more and more negligible when τ increases. This may occur if the suspending medium viscosity, η_f, or the particle radius, a, increases. Conversely, if the difference between the particle and suspending medium density increases or if ω increases, τ will decrease and the centrifugal shift will be more important. Increasing ω or η_f will also reduce the radius of the orbit, whereas increasing the density difference (ρ_p -ρ_f) and the particle radius, a, will increase u_p, and thus the circular path radius.

The particle motion X(t) and Y(t) in the ground-based frame may be easily obtained from Equ. (15) and (16):

$$X(t) = \frac{u_p}{\omega} + \left(x_0 - \frac{u_p}{\omega}\right) e^{\frac{t}{\tau}} \cos(\omega t) - y_0\, e^{\frac{t}{\tau}} \sin(\omega t) \quad (17).$$

$$Y(t) = \left(x_0 - \frac{u_p}{\omega}\right) e^{\frac{t}{\tau}} \sin(\omega t) + y_0\, e^{\frac{t}{\tau}} \cos(\omega t) \quad (18).$$

Since $$(X(t) - \frac{u_p}{\omega})^2 + Y^2(t) = \left[\left(x_0 - \frac{u_p}{\omega}\right)^2 + y_0^2\right] e^{\frac{2t}{\tau}} \quad (19),$$

one can recognize a circle with an increasing radius and a stationary center. The rotation along this circle is counter-clockwise. The physical parameters influencing the particle trajectory remain the quantity (u_p/ω) and the centrifugal time τ.

4. Discussion and conclusion

The particles trajectories predicted by Equ. (15) and (16) as well as Equ. (17-18) are in excellent agreement with the experimental results of Pollack et al. [5] and Wolf and Schwarz [6]. Pollack et al. [5] observed that, in the rotating frame of reference, microcarriers with density greater than the surrounding medium followed a circular motion relative to the culture medium combined with a migration towards the outer wall of the reactor. Their results confirm that the microcarriers sedimentation velocity does not depend on ω, and is the same as in free fall conditions.

Experiments by Wolf and Schwarz [6] examined parameters (gravitational strength, fluid rotation rate, particle sedimentation rate, and particle initial position) within the useful range for tissue cultures in NASA rotating wall culture bioreactors, and they observed that the rotating fluid effectively counters sedimentation. Results from this group demonstrate that the speed of the particle motion through the rotating fluid medium is the same as its terminal sedimentation rate through a stationary fluid (for identical gravitational conditions). They also demonstrate that the diameter of the nearly circular path is reduced for the lower sedimentation rate, and that it is increased for augmented gravitational acceleration. They show that increasing the angular rotation rate from 8.64 r.pm. to 17.7 r.p.m. induces a reduction of the diameter of the particle path.

In tissue engineering applications, the size of the suspended particles may change during the culture due to cell proliferation and/or recruitment of additional cells into an aggregate, causing an increase in sedimentation velocity by the square of the radius. To counteract the increase in sedimentation velocity, the speed of rotation may be augmented. However, a low shear environment has to be maintained during cell cultivation (especially in recellularization experiments). In order to minimize mechanical damage to cultured cells, optimal setting of the peristaltic pump is required: choice of the tubing, low pump motor speed, minimized occlusion by the roller heads, … Complete filling of the chamber and solid body rotation of the culture medium should also be achieved. The fluid thus rotates at the same angular velocity as the chamber walls and thereby creates a laminar flow with minimal shear force. From a theoretical point of view, the mathematical descriptions presented in this paper are valid when the spheres and the rotation rate are sufficiently small so that viscosity dominates and the Reynolds numbers remain small. Complete filling of both **Part A** and **Part B** of our device minimizes the influence of the deformability and porosity of the esophagus wall, that are not taken into account in the present theoretical analysis.

The length of the tissue construct is important in such type of devices: the longer it will be, the lower will be the relative importance of entry and exit flow perturbations. A numerical study of the flow inside the RCCSmax bioreactor would allow to describe more precisely the esophagus entry and outlet area (possible flow stagnation or cell accumulation).

In conclusion, the RCCS Synthecon bioreactor appears to be a convenient device for cell culture and esophagus tissue engineering since it allows controlled mechanical stimulation, through the combination of flow perfusion and rotation. Cells or particles are constantly maintained in suspension

in the media, which insures that nutrient, oxygen, and waste transfer will not be limited by diffusion as they are in static culture systems. Forces that might damage cells are minimized in this device and low shear stress environment is created provided that the perfusion rate and the rotation speed remain moderate ($\omega < 20$ rpm and $Q < 30$ ml/min).

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