Development of Blood Analog Fluids Using Human Hair Protein Particles*

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Model experiments of blood flow are very important in the study of mechanical aspects in cardiovascular research and the development of artificial organs. Several blood analog fluids, such as non-Newtonian fluids have been developed and used in model experiments. However, little is known about blood substitutes with biocompatible properties. We have developed novel procedures for preparing human hair protein films, and have fabricated protein particle suspensions from the films, by mechanical stimulation, for use as blood analog fluid. The average diameter of the protein particles was controlled and microscopic observations were done using a confocal microscope. The Casson's plot patterns of the suspension containing the protein particles were similar to those of human blood. The protein particles also worked well as ultrasound contrast agents in the ultrasound Doppler flow velocity measurements in the model experiments. Therefore, the protein particle system is a promising alternative for blood cells in artificial blood.

Key Words: Human Hair Protein, Particle Preparation, Blood Analog Fluids, Biomechanics

1. Introduction

Cardiovascular disease is the primary cause of morbidity and mortality in the western world. It has been suggested that it will become the leading cause of death worldwide in the 21st Century. To clarify the disease from the aspect of mechanics such as hemodynamics, *in vitro* model experiments of blood flow are very important. In addition, *in vitro* experiments of artificial organs such as an artificial heart are also important for their development. Using blood is ideal for *in vitro* model experiments, but there are problems in its use: for example, (1) individual differences in blood, (2) putrefaction in long term experiments, (3) hazard of infectious diseases. A blood analog fluid as a substitute for blood is required for *in vitro* model experiments. Glycerol solution has generally been used in experiments, but it is a simple Newtonian fluid, whereas the blood is a non-Newtonian fluid consisting of cells and plasma. Several blood analog non-Newtonian fluids have been developed and used in *in vitro* model experiments. Ohba et al.[1] developed a model blood consisting of a dense suspension of alginic acid calcium gel particles. Nguyen et al.[2] proposed a simple method of simultaneously matching the refractive index and kinematic viscosity of a circulating blood analog in hydraulic models for optical flow measurement techniques. However, little is known about the long-term stability and biocompatibility of blood substitutes for future applications as artificial blood and carriers for drug delivery systems.

We found that particles and filamentous structures with a diameter of $1-3\,\mu m$ are contained in human hair protein films[3]. From wet hair protein films, we prepared protein particle suspensions with an average diameter of $3-45\,\mu m$ by mechanical stimulation. Consequently, we focused on red blood cells in blood and adapted the hair protein particles as a model of red blood cells, and developed protein particle suspensions for use as blood analog fluids. The following are the advantages of the hair protein solutions as blood analog fluids. (1) Since blood cells also consist of protein, the protein particles are expected to have the same properties as red blood cells. (2) Low cost and mass production are possible. (3) Protein particle

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suspensions can be stored for extended periods of time of up to 8 months.

In the first stage of the development of the blood analog, we prepared protein suspensions as non-Newtonian fluid, and used them in *in vitro* experiments.

2. Materials[3]

Human hair was washed in ethanol and then incubated with "Shindai solution" consisting of 25 mM Tris-HCl, pH 8.5, 2.6 M thiourea, 5 M urea and 5% 2-mercaptoethanol at 50°C for 2–4 d. After filtration, the solution was further centrifuged at 15 000 g for 10 min at 25°C and the supernatant was used as the starting protein solution for the protein films.

The hair protein solution was exposed to a solution containing $MgCl_2$. After standing for 1–2 h at room temperature, a membranelike protein aggregate (film) was formed, which was then washed by rinsing with water for over 12 h and then with distilled water for 3 h. The film was crushed by a homogenizer using a dispersing generator operating on the rotor/stator principle (Polytron PTA10SK, KINEMATICA AG, Rotor diameter 9 mm, Stator diameter 12 mm). The rotor speed of the homogenizer can be changed. Then we added carrier fluid (distilled water or gellan solution) to the crushed protein to form the blood analog fluid. The reasons of using gellan solution as the carrier fluid are as follows: (1) gellan is a biocompatible material, and (2) gellan solution is better than glycerol solution for *in vitro* experiments of the PVA hydrogel model vessel[4]. Table 1 shows the change in size of PVA hydrogel tubes in gellan and glycerol solutions (viscosity: 5 mPa·s) with time. PVA hydrogel did not shrink in gellan solution over a long time.

3. Methods

First, the protein film was homogenized for 5 s (rotor speed: 8 500 rpm) and used as the "original suspension". Then the suspension was crushed using a homogenizer for 300 s with rotor speeds of 8 500, 19 000, 23 000, and 26 000 rpm. The diameter and its distribution were measured using a laser diffraction particle size analyzer (Shimadzu SALD-200V). The shape and size of the protein particles were observed using a confocal microscope (BIO-RAD, Radiance2000). The confocal microscope was better than the optical microscope and the scanning electron microscope for seeing the three-dimensional shape in fluid (e.g., water). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 13.5% polyacrylamide gel.

The viscosity of protein particle suspension (0.1 and 0.4 mg/mL) was measured using a cone and plate viscometer at 25°C with various shear rates. Shear stress was also measured. The carrier fluid of protein particle suspension was distilled water or gellan solution (0.25 wt%).

The protein suspension was perfused through a model of a diseased artery made of PVA hydrogel[4]. Pressures and flow rate upstream and downstream of the model were measured using pressure-transducers and electromagnet flow meters. The flow velocity was measured using a Doppler ultrasound scanner (Medison 6000CMT).

Table 1 Change in the length of PVA hydrogel tubes in gellan and glycerol solutions (viscosity: 5 mPa·s) with time. PVA hydrogel did not shrink in gellan solution over a long time

	0 h (initial length)	12 h	24 h
Gellan solution	53 mm	53 mm	53 mm
Glycerol solution	53 mm	48 mm	47 mm

4. Results and Discussion

4.1 Particle diameter and morphology

Figure 1 shows the particle diameter distribution of original suspension (homogenizing time: 5 s; rotor speed: 8 500 rpm). This shows there are large particles from 70–300 μm in the suspension and the average diameter of the protein is approximately 130 μm. The relationship between the average particle diameter and homogenizing time is shown in Fig. 2. The average diameter decreased with an increase in homogenizer rotor speed and converged to a certain value with homogenizing time over 100 s. This figure also shows that the average particle diameter depends on the homogenizer rotor speed than homogenizing time. Figure 3 shows the relationship between homogenizer rotor speed and average particle diameter of the protein particles. From these figures, we found that the average diameter after homogenization is controlled because the relationship between average particle diameter of the protein particles and homogenizer rotor speed is approximated to an exponential function. The rotor speed of 19 000 rpm will be a favorable value for producing particles with the same diameter as red blood cells (approx. 8 μm). Figure 4 shows the particle diameter distribution of suspension after 300 s of homogenization (rotor speed: 19 000 rpm). The shape of the distribution is different from

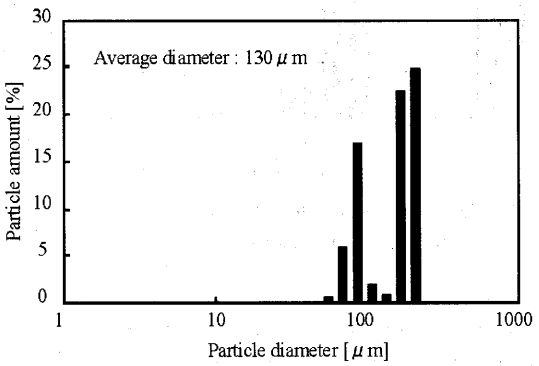


Fig. 1 Particle diameter distribution of suspension (Homogenizing time: 5 s, Rotor speed: 8 500 rpm)

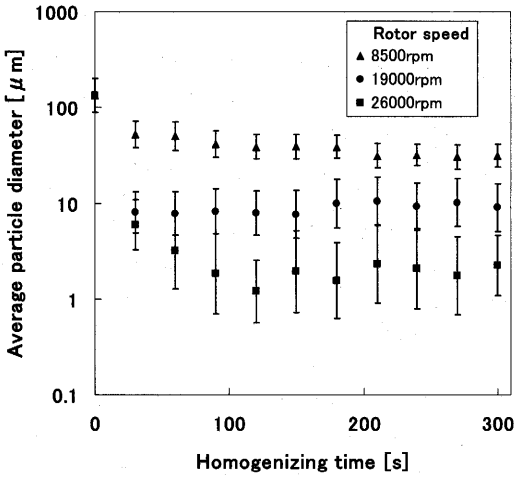


Fig. 2 Relationship between homogenizing time and average particle diameter. Error bars show standard deviation based on logscale particle diameter distribution

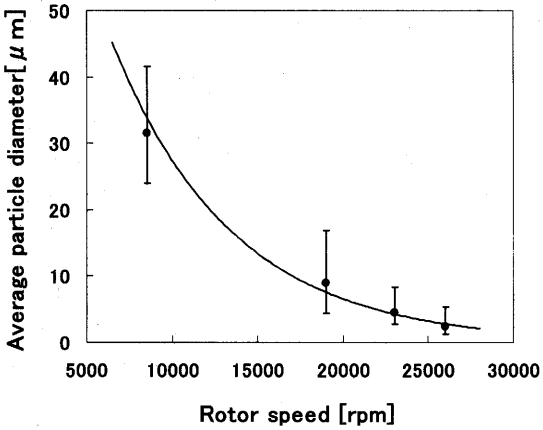


Fig. 3 Relationship between rotor speed of homogenizer and average particle diameter (Homogenizing time: 300 s). Error bars show standard deviation based on logscale particle diameter distribution

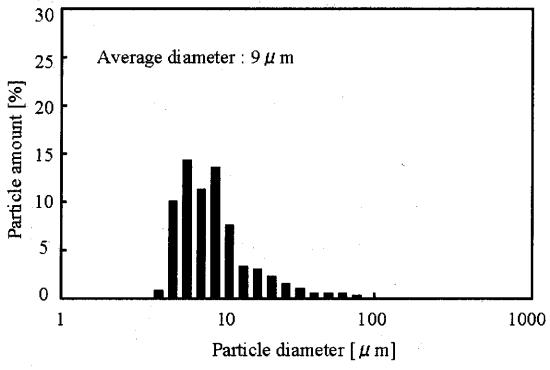


Fig. 4 Particle diameter distribution of suspension (Homogenizing time: 300 s, Rotor speed: 19 000 rpm)

that in Fig. 1 and the average diameter is 9 μm, which is close to that of red blood cells. However, particles greater than 10 μm and smaller than 8 μm also remain. Figure 5 shows a micrograph of the protein particles dyed with rhodamine in distilled water, taken using a confocal microscope. From this micrograph, the particles were seen to be spherical or irregular in shape. Therefore, we could control not the variance and shape of particles but the average diameter. The homogenizer may be limited in milling the particles to the same diameter. Other methods using an ultrasound homogenizer and filtering or centrifugal separation will be needed to obtain monodispersed protein particles.

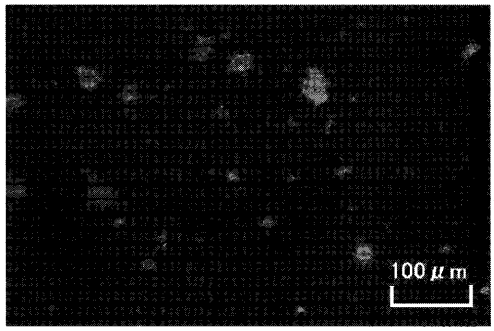


Fig. 5 Confocal microscope image of protein particles in distilled water (Homogenizing time: 300 s, Rotor speed: 19 000 rpm)

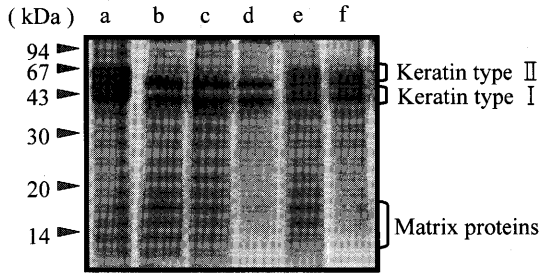


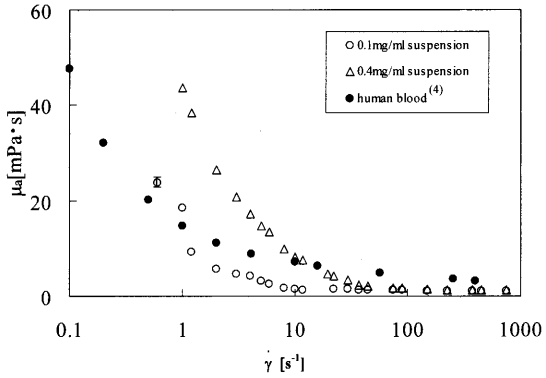
Fig. 6 SDS-PAGE of the protein particle suspension. a, hair protein solution; b, 1 day; c, 1 month; d, 2 months; e, 4 months; f, 8 months

4.2 Protein components and stability

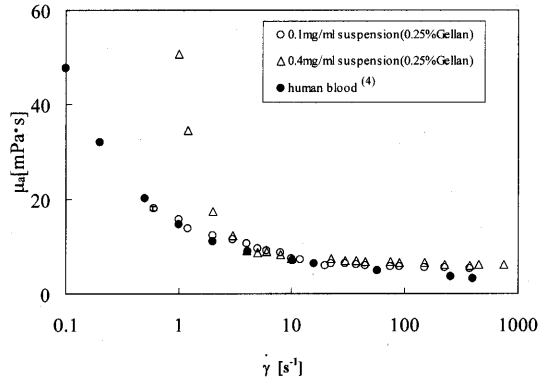
The protein components of the particle suspension were examined by SDS-PAGE (Fig. 6). Original human hair protein solution mainly consisted of α-keratins with molecular mass of 40 – 60 kDa and matrix proteins with molecular mass of 10 – 20 kDa[3]. In the protein particle suspension, α-keratins are present, while the content of matrix proteins was low compared with the hair protein solution. No significant degradation of α-keratin was found after mechanical stimulation. Furthermore, no significant degradation was found in the samples stored for 8 months, indicating that the protein particle suspension is excellent in terms of stability.

4.3 Viscosity of suspension

Figure 7 shows the relationship between shear rate $\dot{\gamma}$ and apparent viscosity of the suspension μ_a and human blood (Ht = 39)[5] for comparison. As the carrier fluid of suspension, water (viscosity: 1 mPa·s) (Fig. 7 (a)) or 0.25 wt% gellan solution (viscosity: 5 mPa·s) (Fig. 7 (b))

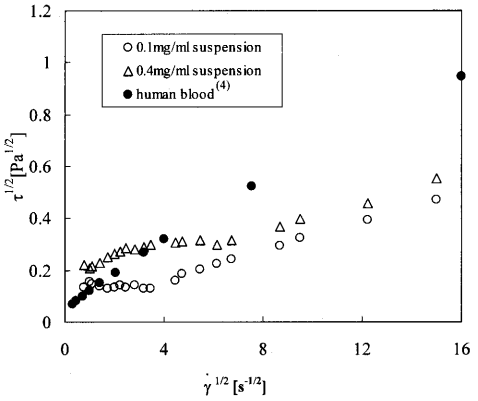


(a) Carrier fluid: water

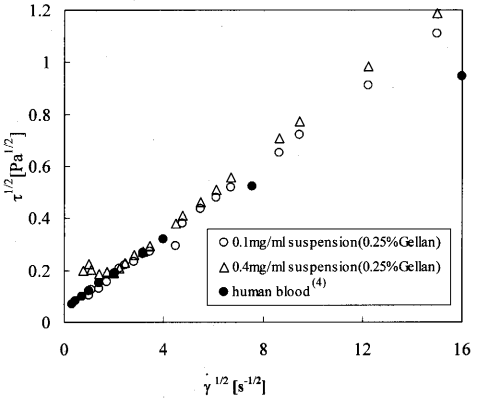


(b) Carrier fluid: 0.25 wt% gellan solution

Fig. 7 Relationship between shear rate and apparent viscosity. Error bars on plots of 0.1 mg/mL suspension for $\dot{\gamma} = 0.6\,s^{-1}$ denote standard deviation ($n = 4$) to represent accuracy



(a) Carrier fluid: water



(b) Carrier fluid: 0.25 wt% gellan solution

Fig. 8 Casson's plot of protein particle suspension and human blood

was used. The suspensions act as non-Newtonian fluid and show the shear thinning phenomenon (reduction of viscosity by increase in shear rate), similar to blood. The apparent viscosity of the suspensions increased with an increase in the concentration of particles. For high shear rates over $50\,s^{-1}$, the apparent viscosity converged to that of carrier fluid. Using gellan solution is effective for altering the apparent viscosity to that of human blood. Figure 8 shows the Casson's plot[6] representation of Fig. 7 as the relationship of the square root of shear rate and square root of shear stress. The plots of the suspensions of 0.25 wt% gellan solutions (Fig. 8 (b)) were almost linear.

As seen from the above two figures, the suspensions of the protein particles also show the same rheological property as human blood. Furthermore, the suspension of 0.1 mg/mL protein and 0.25 wt% gellan solution as carrier fluid is the blood analog fluid closest to human blood. However, for the protein particles as the model of red blood cells, the 0.1 mg/mL protein concentration is much lower than the relative hematocrit of human blood. This is accounted for by low deformability of protein particles: hair protein particles are stiffer than red blood cells[7]. Furthermore, the viscosity of the suspension of rigid particles

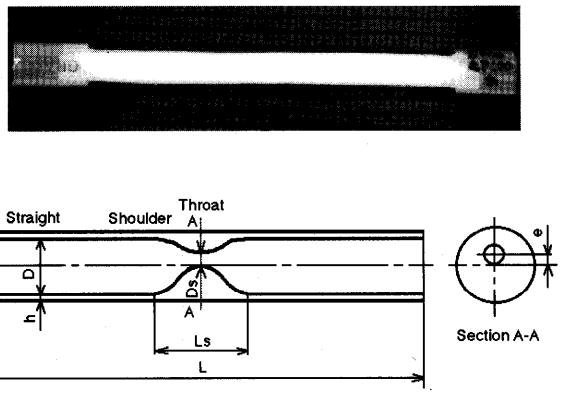


Fig. 9 Photograph and schematic representation of the model of diseased artery with atherosclerosis $L = 110$ mm, $Ls = 16$ mm, $D = 8$ mm, $h = 1$ mm

depends on the volume of particles rather than on the diameter[7]. Hence, the improvement of the low deformability of particles is very important for blood analog fluid, particularly for microcirculation.

4.4 Flow in the model blood vessel

We attempted to use the suspension to an *in vitro* experiment of atherosclerosis. Figure 9 shows the *in vitro* model of diseased artery with atherosclerosis. The model

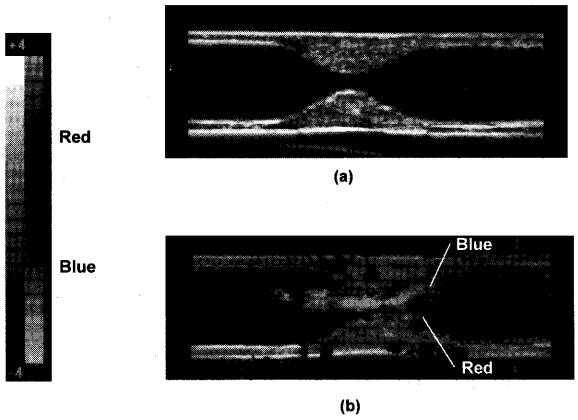


Fig. 10 Ultrasound image of the model with perfusion of the suspension. Steady flow, flow rate: 6 mL/s, probe: linear (7.5 MHz). Flow: Right → Left. (a) B-mode. (b) Color Doppler mode: blue represents downward flow, red represents upward flow; color bar shows velocity of flow (±4 m/s)

is made of PVA hydrogel, and the lumenal diameter of the model is reduced; it is called the stenosis model[4]. Since the inside of the stenosis model is not visible, we used a duplex ultrasound scanner to measure the deformation and flow. Figure 10 shows the ultrasound image of the model while the hair protein particle suspension (concentration of protein particles: 0.2 mg/mL, carrier fluid: 0.25 wt% gellan solution, average diameter of particles: 9 μm) was being perfused through the model. As we described in section 2, this suspension is better than the previous glycerol solutions. In the B-mode image (Fig. 10 (a)), protein particles in the stenosis model are shown. For the Doppler color mode (Fig. 10 (b)), the flow was detected. Upstream of the stenosis, there is downward flow on the upper half-plane and upward flow on the lower half-plane. On the basis of these images, the protein particle is judged to also be a suitable material for ultrasound contrast agents.

5. Conclusion

This study was conducted to develop a suspension of human protein particles and apply it as a blood analog fluid. The following results were obtained.

(1) Average particle diameter was controlled.

(2) Viscosity of the suspension was close to that of human blood.

(3) The protein particles acted as good ultrasound contrast agents.

Problems to be solved in future include the following.

(1) Development of monodispersed protein particles.

(2) Measurement of deformability of protein particles and comparison with red blood cells for blood analog fluid to be used for microcirculation.

(3) Development of the function of the destruction of red blood cells.

Furthermore, as the next future of the development of the blood analog fluid, the function of generation of blood clot will be very important.

Acknowledgement

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