Prevention of Stirring-Induced Microparticle Formation in Monoclonal Antibody Solutions

Tomoyoshi Ishikawa,*[,a,b] Noritaka Kobayashi,[a] Chie Osawa,[a] Eiji Sawa,[a] and Kaori Wakamatsu[b]

[a] *Bio Process Research and Development Laboratories, Production Division, Kyowa Hakko Kirin Company Limited; 100–1 Hagiwara-machi, Takasaki, Gunma 370–0013, Japan: and* [b] *Department of Chemistry and Chemical Biology, Graduate School of Engineering, Gunma University; 1–5–1 Tenjin-cho, Kiryu, Gunma 376–8511, Japan.*
Received November 30, 2009; accepted March 11, 2010; published online March 24, 2010

Monoclonal antibodies are being widely used for the treatment of various diseases. Microparticle formation in high-concentration protein solutions is a major problem during the manufacture of therapeutic monoclonal antibodies, because aggregation leads to fouling of aseptic filters and may lead to an immunogenic reaction in patients. We found that stirring using a traditional bottom-magnetic type stirrer results in extensive and sustained formation of 500-nm diameter protein microparticles arising from shear stress on protein molecules. The antibody solution stirred for only 5 min using this type of stirrer exhibited significant fouling of aseptic filter membranes. In contrast, a top-entering type stirrer did not lead to the formation of microparticles, and the solution did not exhibit membrane fouling even after 30 min of stirring. We conclude that a top-entering type stirrer is more suited for the manufacture of concentrated therapeutic monoclonal antibody solutions.

Key words stirring; monoclonal antibody; aggregation; microparticle; shear stress

In recent years, monoclonal antibodies are being widely used as biopharmaceuticals for the treatment of various diseases such as cancer, cardiovascular disease, inflammatory diseases, macular degeneration, transplant rejection, multiple sclerosis, and viral infections because of their minimal side effects due to their high antigen specificity.[1,2] Unlike other aqueous proteinaceous biopharmaceuticals such as cytokines, antibodies are generally administered as active proteins in large amounts to achieve sufficient therapeutic effects. This requires high antibody concentrations in solutions.[3] The concentrations of antibody solutions for intravenous infusion are typically within the range of 1—20 mg/ml, whereas some solutions for subcutaneous administration may require a protein concentration as high as 100 mg/ml or above. Protein molecules tend to aggregate at such a high concentration, and extreme care must be paid during the manufacture of antibody solutions to avoid protein aggregation. Such aggregation leads to fouling of aseptic filters during their manufacture, and may lead to immunogenic reactions in patients.[4—6]

In the presence of appropriate concentrations of salts that suppress ionic interactions, hydrophobic interactions between the hydrophobic surfaces of protein molecules are the major cause of protein aggregation. Protein denaturation exposes hydrophobic residues buried in the protein core and exacerbates protein aggregation.[7] Protein denaturation is induced by many stresses such as heat, light, shear, air bubbles, exposure to hydrophobic surfaces, and changes in pH or ionic strength of the solution. These stresses must be avoided throughout the manufacture of proteins, as well as during their transportation and administration to patients.[4,8—12] The manufacture of aseptic protein solutions is comprised of 3 main processes. The first process is called formulated bulk preparation. During this process, the protein concentration in the solution is adjusted to a predetermined value, and various excipients are added to produce a formulated bulk. The next process is aseptic filtration. During this process, the formulated bulk, which may be contaminated with bacteria, is typically sterilized by passing it through 0.2-μm pore filters. The final process is filling. During this process, aliquots of the filtrate are filled into suitable containers such as vials. The containers are then sealed tightly using rubber stoppers, caps, *etc.* Even during these seemingly simple processes, various stresses applied may lead to protein aggregation Cromwell *et al*. reported that during the transfer of antibody solutions from one container to the other, the number of particles larger than 2 μm increased with the use of a piston pump, but not a rolling diaphragm pump.[4] Tyagi *et al*. postulated that the stainless steel nanoparticles shed from the pump-solution contact surface induced heterogeneous nucleation of immunoglobulin G (IgG) particles.[13]

There have been very few studies discussing aggregate formation during the formulated bulk preparation process of antibody solution manufacture, in which various excipients such as sodium chloride, phosphate, amino acids, and detergents are added. This process inevitably entails the stirring of antibody solutions. The stirring procedure applies a shear stress to antibodies that can lead to aggregate formation. Aggregates generated during this process make the aseptic filtration step difficult. Although microparticles clog the filters, it is impossible to exchange the clogged filter halfway through the process without risking microbial contamination. Increasing the membrane area may lead to protein adsorption and a low recovery of proteins. Therefore, it is quite important to suppress the generation of protein microparticles at the formulated bulk preparation step. In the present study, we examined protein aggregation during the stirring phase of the formulated bulk preparation process using 2 methods: a conventional stirring method and an improved stirring method. We found that the improved stirring method does not generate microparticles and significantly facilitates the aseptic filtration process.

MATERIALS AND METHODS

Materials The pharmaceutical grade IgG_1 antibodies (IgG-A and IgG-B) used in this study were produced at Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). Their isoelec-

* To whom correspondence should be addressed. e-mail: tomoyoshi.ishikawa@kyowa-kirin.co.jp © 2010 Pharmaceutical Society of Japan

tric points (p*I*) were approximately between 8 and 9. The concentration of each antibody was adjusted to 10 mg/ml in a sodium glutamate buffer (pH 5.5) containing sorbitol and polysorbate 80. All excipients conformed with the United States Pharmacopeia/National Formulary.

Conventional Stirring Method Using Bottom-Sitting Magnet-Driven Vanes Using 2 different sizes of stainless steel tanks, we examined the effects of the conventional stirring method, in which antibody solutions are stirred using vanes fixed on a ring that rotates around a rod at the bottom of the tank (Fig. 1, left). The ring was driven by strong magnets beneath each tank. One was a 10-l capacity manufacturing size tank and contained 7 l of antibody solution. The other was a 7-l capacity laboratory tank, 21.2 cm in diameter with 10.5-cm diameter vanes, and contained 4 l of antibody solution. The solutions were stirred at 50 rpm, and aliquots were sampled at the indicated times up to a period of 30 min.

Improved Stirring Method Using a Top-Entering Stirrer Using a 2-l stainless steel laboratory tank, 15.0 cm in diameter, we examined the effects of a new stirring method by which antibody solutions were stirred using 6.9-cm diameter vanes fixed on the rotating shaft that entered the solution from above (Fig. 1, right). One liter of the antibody solution was stirred at 50 rpm for 30 min, followed by stirring at 150 rpm for 15 min to apply further stress. Aliquots were sampled at the indicated times.

Determination of the Number of Microparticles in Solution The number of invisible microparticles was determined using a liquid particle counting system (HIAC Royco, Hach Company, Loveland, CO, U.S.A.). After injecting 200 μl of each specimen, the number of microparticles in the solutions was counted, and results were obtained for 3 different size ranges of particles: $>2\,\mu$m, $>10\,\mu$m, and $>25\,\mu$m.

Dynamic Light Scattering (DLS) The size distribution of the microparticles in the antibody solutions were determined more precisely using a Zeta Sizer Nano-ZS dynamic light scattering photometer equipped with a He–Ne laser (Malvern Instruments Ltd., Worcestershire, U.K.). Scattered light intensity was detected at an angle of 173°. The samples were analyzed at 20 °C without prior filtration.

Fourier-Transform Infrared (FT-IR) Measurement The FT-IR spectra of the large visible particles formed in antibody solutions were recorded on a Spectrum 100 spectrometer (Spectrum Spotlight 300 microscope system; Perkin-Elmer, Inc., Waltham, MA, U.S.A.). Particles were washed thoroughly with purified water before measurement.

Transmembrane Permeation Test of the Antibody Solution after Stirring The membrane permeability of the antibody solutions was determined using 0.22-μm pore polyvinylidene difluoride (PVDF, 13.8 cm^2) membranes (Millipore, Bedford, MA, U.S.A.). By applying a constant pressure of 50 kPa, 500 ml of the swirled antibody solution was permeated through the aseptic filtration membranes. The filtrate volumes were measured at regular time intervals, and the maximum permeation volume (V_{max}) was calculated by assuming the standard occlusion model.[14)]

Size Exclusion (SEC)-HPLC The amounts of small soluble aggregates and degradation species were analyzed by SEC-HPLC using 2 G3000SWXL columns (7.8 mm i.d.× 30 cm; Tosoh Corp., Tokyo, Japan) connected in tandem. The mobile phase contained 50 mM sodium phosphate (pH 7.0) and 500 mM sodium chloride. The experimental conditions were as follows: injected protein, 20 μg; flow rate, 0.5 ml/min; detection wavelength, 215 and 280 nm; analysis time, 60 min.

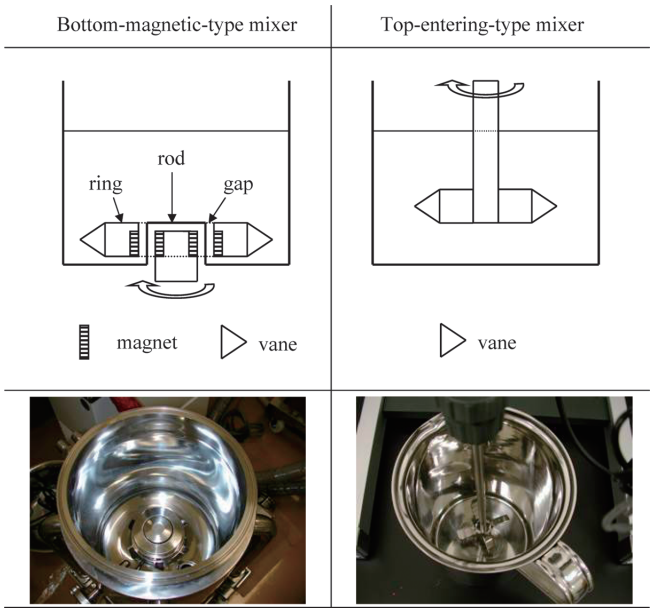

Fig. 1. Configuration of the Stirring Equipment

Bottom-magnetic type stirrer has stirring vanes fixed on a ring that rotates around the rod at the tank bottom. The ring was driven by strong magnets beneath the tank. The top-entering type stirrer has stirring vanes fixed on the rotating shaft and enters the solution from above.

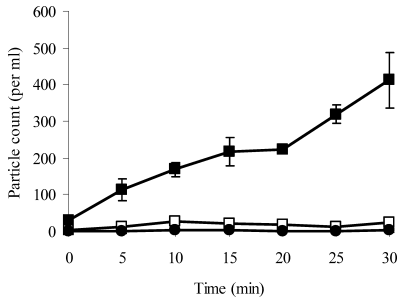

Fig. 2. Time-Dependent Microparticle Formation in an Antibody Solution Stirred Using a Bottom-Magnetic Type Stirrer

An IgG-A antibody solution (10 mg/ml) was stirred using a bottom-magnetic type stirrer in a manufacturing size tank at 50 rpm and room temperature (about 25 °C). Particle sizes are as follows: (■) $>2\,\mu$m, (□) $>10\,\mu$m, and (●) $>25\,\mu$m. Error bars represent standard deviations of triplicate determinations.

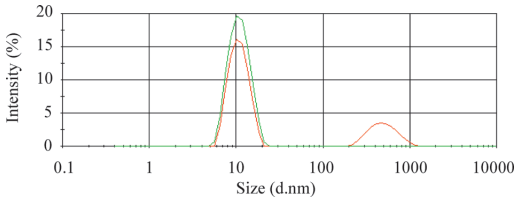

Fig. 3. Particle Size Distribution in IgG-A Antibody Solutions

Green line, antibody solution before stirring; red line, antibody solution after being stirred using a bottom-magnetic type stirrer for 30 min in a manufacturing size tank at 50 rpm.

RESULTS

Characterization of Particles in the Antibody Solution Formed During Stirring in a Manufacturing Size Tank Antibody solutions were stirred by vanes that were driven by a rotating magnet underneath the tank. The effects of this type of stirring method on microparticle generation were assessed first using a 10-l manufacturing size tank.

The antibody solution became turbid as the solution was stirred (not shown) and the number of particles larger than 2 μm increased with time. However, the increase in the number of much larger particles (>10 μm and >25 μm) was not remarkable (Fig. 2). These observations indicated that stress from the conventional stirring method generated microparticles in the protein solution as early as 5 min after stirring was initiated.

The radius of these microparticles was analyzed by dynamic light scattering to characterize the formed particles. Although the sample before stirring exhibited a single peak at about 10 nm as expected from the IgG monomer size,[15] the sample after stirring exhibited an additional peak near 500 nm (Fig. 3) in agreement with the increased particle count of >2 μm (Fig. 2). Note that 2 μm-particles are not detectable by DLS because the concentration of the particles is far lower than that of monomers that exhibit the peak at 10 nm: 500 particles/ml *vs.* 10 mg/ml (4×10^{16} molecules/ml). The FT-IR spectrum of the large visible particles that were formed exhibited absorption specific to amide bonds at 1637 cm^{-1} (amide I) and 1540 cm^{-1} (amide II), which strongly suggests that the microparticles were composed of protein (Fig. 4). The effects of stirring on the generation of small (invisible) soluble antibody multimer aggregates and their fragments were analyzed by SEC-HPLC. No significant changes in the amount of small aggregates and fragments occurred after stirring (Fig. 5). These observations suggest that large aggregates (about 2 μm diameter) were directly formed from antibody monomer and not *via* stable intermediate oligomeric forms. SEC-HPLC also indicated that the fraction of antibody proteins involved in aggregate formation was quite small because the peak area of the antibody monomer did not change significantly with stirring (not shown).

Comparison of the Effects of Stirring Methods on Microparticle Formation To solve the problem of microparticle formation during conventional stirring of an antibody solution, we analyzed top-entering type stirring on microparticle formation and compared it with that of conventional bottom-magnetic type stirring. During bottom-magnetic type stirring, we observed increases in the number of microparticles as stirring time increased (Fig. 6A). We were also able to visually confirm an increase in turbidity. However, no microparticles were generated after top-entering type stirring and no increase in microparticles was noted even after the rotation speed was accelerated threefold (Fig. 6B), and the solution was transparent and colorless. These results confirm

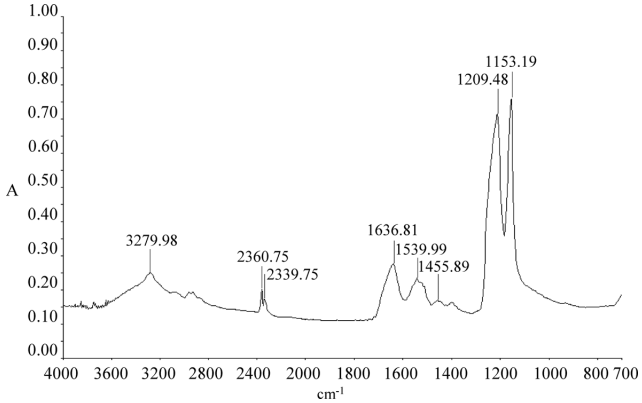

Fig. 4. FT-IR Spectrum of Microparticle in IgG-A Antibody Solution

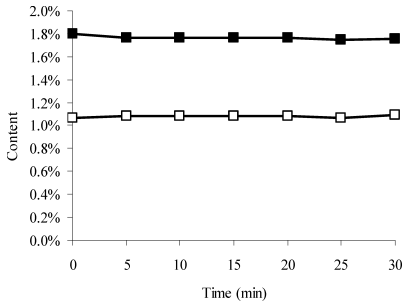

Fig. 5. Fraction of Soluble Aggregates and Degradation Species in an IgG-A Antibody Solution Monitored as a Function of Stirring Time

The antibody solution (10 mg/ml) was stirred using a bottom-magnetic type stirrer in a manufacturing size tank at 50 rpm. Error bars represent standard deviations of triplicate determinations. (■): soluble aggregates, (□): degradation species.

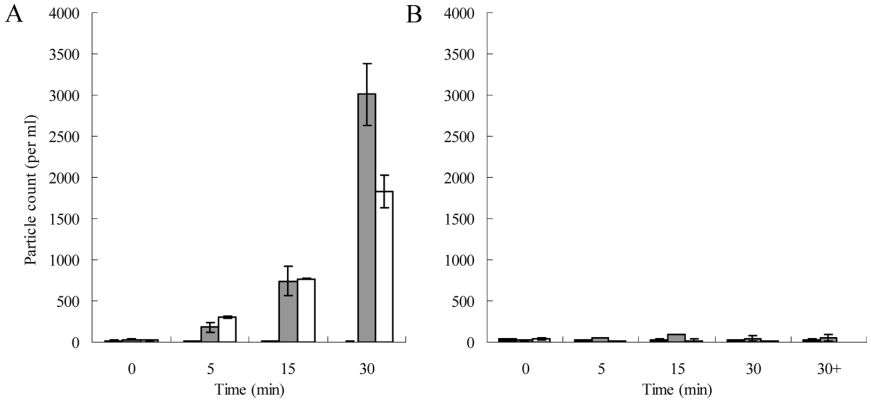

Fig. 6. Particle Formation in Antibody Solutions Stirred by 2 Different Stirrers as a Function of Stirring Time

Black bar (almost indiscernible), protein-free buffer; gray bar, IgG-A solution (10 mg/ml); white bar, IgG-B solution (10 mg/ml). (A) The solutions were stirred using a bottom-magnetic type stirrer at 50 rpm. (B) The solutions were stirred using a top-entering type stirrer at 50 rpm. "30+" indicates a sample that had been stirred for an additional 15 min at 150 rpm after being stirred for 30 min at 50 rpm. Error bars represent standard deviations of triplicate samples.

Table 1. Transmembrane Permeation Test for Antibody Solutions

Stirring time (min)	V_{max} value (l)			
	5	15	30	30+
Bottom-magnetic type				
Protein-free buffer	>30	>30	>30	Not tested
IgG-A	9.2	1.5	0.6	Not tested
IgG-B	9.6	2.3	0.6	Not tested
Top-entering type				
IgG-A	>30	>30	>30	>30
IgG-B	>30	>30	>30	>30

The antibody solution (10 mg/ml) was stirred using a bottom-magnetic type stirrer in a manufacturing size tank at 50 rpm. "30+" indicates that the sample that had been stirred for an additional 15 min at 150 rpm after being stirred for 30 min at 50 rpm. Antibody solutions (approximately 500 ml) that had been stirred for the indicated times were passed through 0.22-μm aseptic PVDF membranes (13.8 cm^2) under a constant pressure of 50 kPa. The filtrate volume was measured at regular time intervals, and the maximum permeation volume (V_{max}) was calculated by assuming the standard occlusion model.[14]

that top-entering type stirring did not generate microparticles. It should also be noted that no significant microparticle formation was noted in a protein-free buffer even when it was stirred using a bottom-magnetic type stirrer. This observation strongly suggests that stainless steel microparticles, which may serve as nuclei for heterogeneous protein particle, were not shed from the stirrer surface.

To confirm the advantage of top-entering type stirring in a more practical setting, fouling of aseptic filters by antibody solutions was analyzed. Protein solutions are generally filter-sterilized through 0.2-μm pore aseptic filters. Microparticles generated by bottom-magnetic type stirring have a diameter close to the membrane pore size and are expected to foul the membranes. Membrane fouling can be evaluated by the V_{max} value, the maximum volume to be filtered, which is calculated by measuring the cumulative volumes filtered at several time points. The V_{max} values determined using PVDF membranes are summarized in Table 1. Although no fouling was detected using buffer alone, there was a considerable decrease in the V_{max} value for antibodies IgG-A and IgG-B when they were stirred for 5 min by the bottom-magnetic type stirrer. The V_{max} value decreased with a longer stirring time. In contrast, the V_{max} values of both antibodies stirred using the top-entering type stirrer did not exhibit any decrease after stirring for 30 min. V_{max} values did not decrease even after additional stirring for 15 min at a threefold accelerated rate.

DISCUSSION

Tyagi *et al.* found that stainless steel nanoparticles shed from the solution contact surfaces of the pump that was used to fill vials induced heterogeneous nucleation of antibody protein.[13] They reached this conclusion because the protein-free buffer, which was pumped with a positive displacement piston pump having a stainless steel head, induced the formation of protein aggregates when added to unpumped antibody solutions. In addition, they noted that the number of microparticles in the added protein solution was 20 times higher than that in the pumped protein-free buffer. In the present study, the microparticles formed after stirring using a bottom-magnetic type stirrer are speculated to be induced by the shear stress applied to antibody molecules that occasionally entered the gap between the ring and the rod at the bottom of the tank, rather than by the microparticles shed from the stirrer surface. Furthermore, no significant microparticle formation was noted in the protein-free buffer that had been stirred using a bottom-magnetic type stirrer (Fig. 6).

Stirring speed also affects microparticle formation in antibody solutions. Wan *et al.* found that during ultrafiltration of antibody solutions, the retentate became visibly cloudy within the first 2 h of operation when the stirring speed exceeded 600 rpm. However, the retentate remained clear even after 6 h by limiting the stirring speed to 300 rpm.[16] In the present study, the stirring speed was set at 50 rpm for safety. When stirred by a bottom-magnetic type stirrer, extensive microparticle formation was observed even at this speed.

The top-entering type stirrer is generally useful for safe stirring of protein solutions other than those of antibodies, such as cytokines, to obtain microparticle-free transparent solutions. Although it is well known that chromatographic resin slurry should not be stirred by magnetic stir bars at the bottom of a container to prevent the crushing of resin particles, the risk of stirring protein solutions using (bottom) magnetic stirrers has not yet been recognized. Our results should be informative to many researchers and engineers who require the safe handling of protein solutions.

In conclusion, we found out that stirring a monoclonal antibody solution using a traditional bottom-magnetic type stirrer resulted in the generation of protein microparticles, which eventually fouled the aseptic filter membranes. However, no microparticle formation or membrane fouling occurred when the solution was stirred using a top-entering type stirrer. Stirring protein solutions using a top-entering stirrer should be widely beneficial for producing protein biopharmaceuticals.

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