Original Article

DOI: 10.5582/ddt.2019.01090

Janus microspheres for enhanced enteral drug delivery: Preparation and orientated attachment to a Caco-2 monolayer

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Summary **Conventional oral preparations generally release incorporated drugs omnidirectionally, including into the lumen, leading to a low bioavailability of drugs that are unstable in the gastrointestinal tract. Here, we designed Janus microspheres for efficient mucosal drug delivery as single-sided-release microspheres with the oriented attachment to mucus and evaluated their attachment to and orientation on a Caco-2 (human Caucasian colon adenocarcinoma cell line) monolayer. The microspheres comprised a mucus-oriented hemisphere of an ammonioalkyl methacrylate copolymer and a protective hemisphere of a hard fat. Fluorescein isothiocyanate-dextran with an average molecular weight of 3,000-5,000 Da (FD4) was used as a model hydrophilic drug. A water-in-oil emulsion-type solvent evaporation method was employed for fabrication of the Janus microspheres. The yield of Janus microspheres was found to be dependent on the polymer-to-hard fat ratio, with a maximum yield of over 90% being obtained at a ratio of 1:2, whereas lower and higher ratios resulted in monolithic or star-shaped microspheres. FD4 was specifically localized in the polymeric hemisphere. A cell culture study revealed that the Janus microspheres attached to a Caco-2 monolayer *via* their polymeric hemispheres with the hard fat hemisphere providing a protective sealing. This may lead to the development of an effective enteral drug delivery system for biomedicines, such as polypeptides and nucleic acids.**

Keywords: Single-sided-release microspheres, hard-fat, ammonioalkyl methacrylate copolymer, solvent evaporation method

1. Introduction

Although oral drug preparations are the most patient-friendly dosage forms because of their convenience, for drugs that are unstable in the gastrointestinal tract and/or show low permeability in the epithelium, the preparation of oral dosage forms remains difficult. Most biomedicines, including polypeptides, such as hormones, cytokines, and antibodies (*e.g.*, insulin and interferon), or nucleic acids, such as antisense RNA, small interfering RNA (siRNA), and microRNA, have these features. Thus, many researchers in the pharmaceutical field have focused on improving the intestinal absorption of biomedicines.

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The use of additives, such as enzyme inhibitors (*1-4*), membrane permeability enhancers (*5,6*), and tight junction modulators (*7-9*), is one of the major strategies used to improve intestinal drug absorption. Alternatively, microparticles have been reported to improve the intestinal absorption of insulin (*7,10,11*) and siRNAs (*12,13*). Although micro- and nano-particles can protect biomedicines against chemical and enzymatic degradation, conventional particulate systems generally show omnidirectional release of a loaded drug. Drugs released into the luminal bulk fluid or content have a higher risk of enzymatic degradation than drugs released directly at the absorptive epithelial surface. In addition, the fluid or contents in the lumen can dilute drugs, causing loss of driving force across the mucosal membrane. Thus, effective drug delivery systems for biomedicines should release the loaded drug only in the vicinity of and toward the epithelial surface.

Some studies have shown that degradation of

peptides in the intestine can be suppressed by preventing their dilution. Sinko *et al.* demonstrated that restriction of the dilution of calcitonin and an organic acid additive by localization in a limited portion of the intestine *via* a delivery system improved the intestinal absorption of this peptide drug (*14*). Maintaining a lower pH at the site of absorption can inhibit the activity of enzymes. Moreover, Takada and coworkers developed a novel hemispherical patch system (GI-MAPS) for peptide drugs. This system releases a drug only from the flat bottom face but not from the spherical body surface, which is coated with ethyl cellulose, an insoluble polymer, thereby limiting the direction of release (*15,16*). In addition, to maintain a high drug concentration near the site of absorption, the flat face of a hemisphere is adhesive to the mucosal membrane and plays a role in attaching the system to the mucosal surface. In contrast, the coated surface of the spherical body or drug reservoir can prevent drug release toward the lumen and protect a drug from attack by luminal enzymes. It has been demonstrated that the intestinal absorption of peptide drugs, such as granulocyte-colony stimulating factor (G-CSF) (*17*) and interferon (*18*), is markedly improved by using GI-MAPS. Thus, GI-MAPS appears to be a unique and attractive intestinal delivery system for biomedicines. However, the fabrication of micro- or nano-sized GI-MAPS or hemispherical preparations remains technically challenging. Hemispheres are produced using several fabrication processes that may require manufacturing systems that are entirely different from those currently used for conventional preparations, and would therefore not be cost-effective to produce.

Janus microspheres are heterogeneous particles composed of two distinct hemispheres and have been proposed for use in various applications, including those for catalysts (*19,20*), imaging agents/nanosensors (*19,21*), and cosmetics (*19*). Several methods, including templating methods (*22*), colloidal assembly (*23*), particle lithography techniques (*24*), glancing-angle deposition (*25*), and capillary fluid flow (*26*), have recently been proposed for the fabrication of Janus microspheres.

Janus microspheres have also attracted the attention of researchers in the field of drug delivery systems. Pulmonary delivery of hydrophilic and hydrophobic anticancer drugs has been reported using Janus maicrospheres (*27*). Janus microspheres show unique properties that are lacking in conventional homogeneous particles, which inspired us to apply these particle in an attempt to improve drug absorption in the intestine. Namely, Janus microspheres can be designed such that a drug is localized in a particular hemisphere and released from a single side.

In the present study, we investigated the feasibility of fabricating Janus microspheres as an enteral delivery system for biomedicines, which are designed to be oriented on mucus and release the encapsulated drug only to mucus, and confirmed the designed properties on a Caco-2 (human Caucasian colon adenocarcinoma cell line) monolayer. The results obtained, *i.e.*, the high efficiency of Janus particle production, the localization of a drug in a specific hemisphere, and the fixed directional attachment to the epithelium, should guarantee the feasibility of focusing drug release on the site of absorption, potentially leading to an increased bioavailability of medications that are poorly absorbed and unstable in the intestine. These results will indicate the utility of Janus microspheres in developing an enteral delivery system for biomedicines.

2. Materials and Methods

2.1. *Study design and approach*

This study was designed with the aim of developing novel Janus microspheres for enteral drug delivery. To achieve this purpose, we selected a biocompatible cationic polymer (ammonioalkyl methacrylate copolymer: Eudragit® RS100) and a hard fat (Suppocire® AM) containing a mixture of glycerides (mainly triglycerides) as materials for Janus particle fabrication. The cationic polymeric hemisphere was expected to be oriented to the intestinal mucosa and release a loaded hydrophilic drug only toward the mucosal side, whereas the glyceride hemisphere would function as a hydrophobic backing to protect against both the leakage of the drug to the luminal side and enzymatic attack from the luminal side (Figure 1). For the fabrication, we adopted a solvent evaporation method using similar conditions to those previously reported to have produced stable Janus microspheres comprising poly

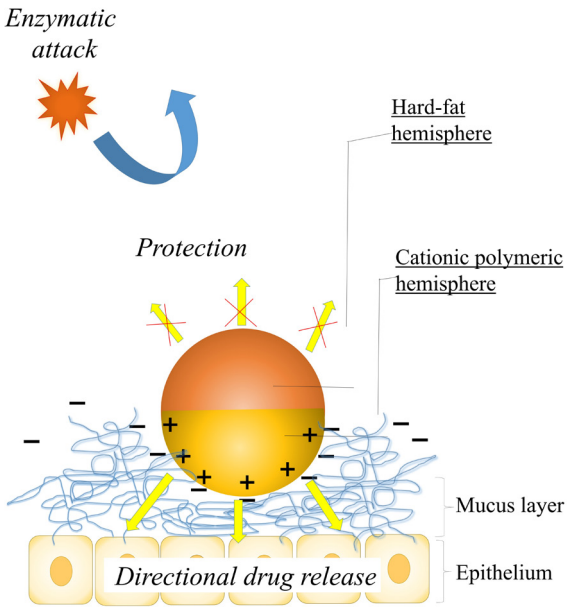

Figure 1. Strategy for promoting drug absorption using single-side releasing microspheres.

(DL-lactide-co-glycolide) and a hard fat (Suppocire AM) (*28*). We use fluorescein isothiocyanate-dextran with an average molecular weight of 3,000-5,000 Da (FD4) as a model drug because biomedicines such as polypeptides and nucleic acids are generally hydrophilic linear macromolecules.

2.2. *Materials*

Ammonioalkyl methacrylate copolymer (Eudragit® RS100) was purchased from Evonik Industries AG (Essen, Germany). The hard fat (Suppocire® AM pellets) was a kind gift from Gattefossé (Lyon, France). Fluorescein isothiocyanate-dextran with an average molecular weight of 3,000-5,000 Da (FD4) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Polyvinyl alcohol (PVA; POVAL 220C) was obtained from KURARAY Co., Ltd., (Tokyo, Japan). Ethoxylated hydrogenated castor oil 60 (HCO60) and caprylic/capric triglyceride (Triester F-810) were supplied by Nikko Chemicals Co., Ltd. (Tokyo, Japan). Tripalmitate glyceride was purchased from Alfa Aesar (Lancashire, UK). Caco-2 cells (RCB0988) were provided by Riken BioResource Research Center (Tukuba, Japan) through the National Bio-Resource Project of the MEX. CellTracker™ Violet BMQC (2,3,6,7-tetrahydro-9bromomethyl-1H,5H-quinolizino [9,1-gh] coumarin) Dye was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Dulbecco's Modified Eagle's medium (D-MEM) and Dulbecco's phosphate buffered saline (D-PBS) were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Fetal bovine serum was purchased from Biowest SAS (Nuaillé, France). All other chemicals used were of reagent grade.

2.3. *Phase diagram analysis*

Mixtures of Eudragit RS100 and Suppocire AM at various ratios were dissolved in methylene chloride. The solvent was evaporated slowly at 20-23°C until phase separation was observed. The resulting solution at this point of phase evaporation was weighed to calculate the concentrations of polymer and triglyceride.

2.4. *Fabrication of Janus microspheres*

Janus microspheres were fabricated using a water-in-oil-in-water (w/o/w)-type emulsion solvent evaporation method (Figure 2a). Eudragit RS100 and Suppocire AM (total amount: 180 mg) were dissolved in methylene chloride (1.5 mL) to prepare the oil phase. Oil Red O or Nile Red (1 mg) was then added to the oil phase as needed. When FD4 was loaded in Janus microspheres, 40 mg/mL FD4 solution (50 µL) as an inner water phase was emulsified into the resulting oil phase for 1 min at 20 kHz, 200 W, and 25% amplitude using a tip-sonicator (Digital Sonifier 250; Branson Ultrasonics Corporation, Danbury, CT, USA) under ice cooling to prepare a water-in-oil (w/o) emulsion. HCO60 (with 1% final concentration in the inner water phase) was added to the inner water phase as needed. The resulting oil phase or the w/o-type emulsion was emulsified into 0.1% PVA-0.1 M phosphate buffer (pH 8.0; 3 mL) at 3,000 rpm using a homogenizer (ULTRA-TURRAX T18, IKA®-Werke GmbH & CO. KG, Staufen, Germany) for 5 min at 20-23°C. The entire resulting w/o/w type emulsion was added to 100 mL of 0.1 M phosphate buffer (pH 8.0), and the diluted emulsion was stirred at 20-23°C for 180 min to remove the solvent. This process is hereinafter referred to as the solvent evaporation process. The hardened microspheres thus obtained were sieved through 149-µm sieves to remove aggregates, washed, and lyophilized. The FD4 content of the resulting Janus microspheres was determined to be 0.500 ± 0.080% (mean ± S.D. n = 3 batches) using the method described in section 2.7 below.

2.5. *Microscopic observations*

During the solvent evaporation process, the emulsion and fabricated Janus microspheres were observed using an optical microscope system (Motic PA300; Shimadzu Co., Ltd., Kyoto, Japan). Fifty microspheres were randomly selected and classified into four conformation types: Janus, double-walled, star-shaped, and monolithic types. The microspheres with each conformation were then counted in triplicate.

2.6. *Determination of Janus particle size*

The sizes of the fabricated Janus microspheres were determined using a laser diffraction particle size analyzer (SALD-2200; Shimadzu Co., Ltd., Kyoto, Japan).

2.7. *Determination of FD4 contents in Janus microspheres*

Janus microspheres loaded with FD4 (10 mg) were weighed in a test tube. The weighed Janus microspheres were dissolved in methylene chloride (2 mL), to which was added 0.05% Tween 80-JP second fluid for the subsequent dissolution test (pH 6.8; 5 mL), and FD4 was extracted by shaking overnight. After centrifuging for 5 min at 2,000 rpm, the upper aqueous layer was collected, and the concentration of FD4 in the aqueous layer was determined using a hybrid multimode microplate reader (Synergy H4; BioTek Instruments, Winooski, VT, USA) based on a fluorescent method. The test was performed in triplicate.

2.8. *Dissolution test*

Janus microspheres loaded with FD4 (10 mg) were weighed in a test tube, to which 0.05% polysorbate 80 (Tween80)-JP second fluid (pH 6.8; 5 mL) maintained at 37°C was added as the dissolution medium, and the microspheres were dispersed. The preparations were immediately shaken at 100 rpm at 37°C in an air-conditioned incubator (BioShaker V-BR-36; TITEC, Koshigaya, Japan) and collected at predetermined times. After centrifugation for 1 min at 2,500 rpm, the medium (0.5 mL) was collected as a sample for assay, and fresh medium (0.5 mL) was added into the tubes. The dissolution test was then continued and performed in triplicate. FD4 remaining after the dissolution test was extracted and determined using the method described in section 2.7. The concentration of FD4 in the samples collected for assay was determined using a fluorescence spectrometer.

2.9. *Differential scanning calorimetry (DSC) analysis*

The microspheres (10 mg) prepared from Eudragit RS100 alone, Suppocire® AM alone, or Eudragit® RS/Suppocire® AM (1:2 blend; unloaded FD4) were analyzed using differential scanning calorimetry (DSC-60; Shimadzu Co., Ltd.).

2.10. *Observation of Janus microspheres on Caco-2 cell monolayers*

Caco-2 cells were cultured in D-MEM containing 10% fetal bovine serum in six-well plates (Corning Inc., New York, USA), each well of which contained 5 mL of the medium. The cells were grown for several days until a confluent monolayer had developed. Caco-2 monolayers were stained with CellTracker™ Violet BMQC Dye (at a final staining concentration of 25 μM). Janus microspheres encapsulating FD-4 and Nile red were suspended in 0.1 M phosphate buffer (pH 7.9). A 400-μL aliquot of the resulting suspension containing 6.3×10^4 particles was then added to the apical side of each well. When all particles precipitate at the bottom of the well, a concentration of 6.3×10^4 particles is equivalent to 7.0×10^3 particles/cm^2. After overnight incubation, the apical side of the well was carefully washed twice with the pre-warmed D-PBS. To evaluate the orientation of attachment of the particles, confocal images were taken under an LSM 510 META microscope (Carl Zeiss Microscopy Ltd, Jena, Germany).

Given that it has previously been suggested that long-term agitation and incubation may cause an increase in the formation of large aggregates and the attachment of microtubes onto B-cells (*29*), incubations in the present study were carried out without agitation to avoid the formation of aggregates or agglomerates, since it is difficult to evaluate their orientation.

3. Results

3.1. *Phase diagram analysis*

Phase separation was observed in the ammonioalkyl methacrylate copolymer, triglyceride, and methylene chloride ternary system. The critical concentration of phase separation showed an approximately negative correlation between the ammonioalkyl methacrylate copolymer and triglyceride concentrations (Figure 2b). The solution at the critical concentration showed a higher viscosity at higher ratios of ammonioalkyl methacrylate copolymer to triglycerides. Under the conditions of a copolymer-to-triglyceride ratio of 1:1, phase separation was observed at 10% (w/w) when

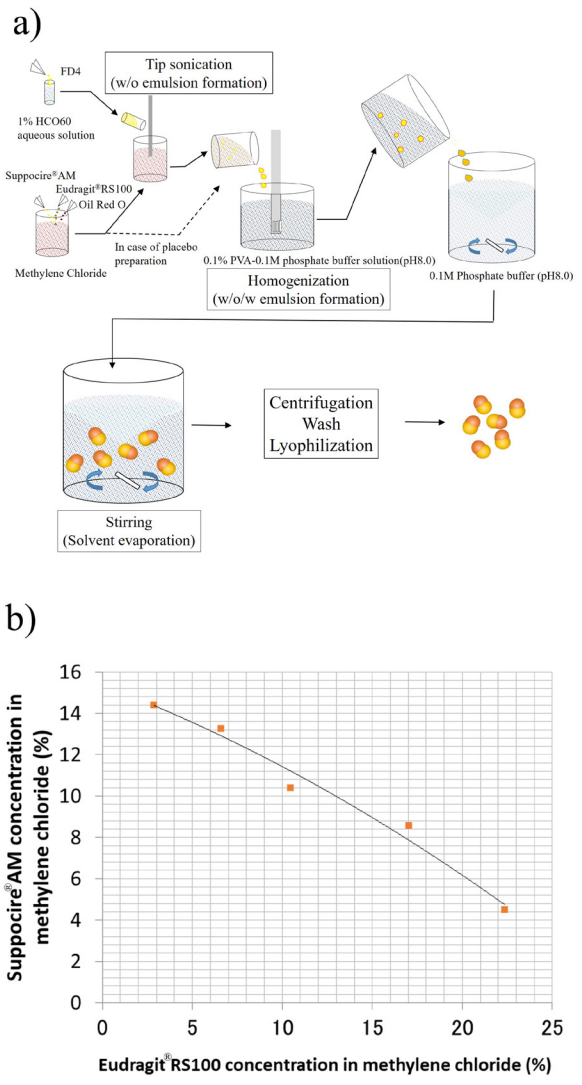

Figure 2. Fabrication of Janus microspheres using a phase separation method. (a) Scheme of the procedure used to fabricate Janus microspheres. **(b)** Phase diagram of the ammonioalkyl methacrylate copolymer (Eudragit® RS100) - triglycerides (Suppocire® AM) - methylene chloride ternary system.

Suppocire AM was used as the triglyceride, whereas it was detected at 23% (w/w) and 14% (w/w) for Triester F-810 and tripalmitate glyceride, respectively.

3.2. *Effects of ammonioalkyl methacrylate copolymer/triglyceride ratio on the conformation of microspheres*

The optical micrographs of microspheres prepared using different ratios of Eudragit RS100 to Suppocire AM are shown in Figure 3. The nonpolar dye Oil Red O can stain the triglycerides in microspheres. Microspheres of triglycerides alone showed a double-walled conformation. This conformation changed to that of Janus and star-shaped microspheres as the ammonioalkyl methacrylate copolymer ratio increased. The microspheres fabricated from 90% ammonioalkyl methacrylate copolymer showed a monolithic conformation and had the same conformation as microspheres prepared from ammonioalkyl methacrylate copolymer alone. The frequency of each conformation corresponding the ammonioalkyl methacrylate copolymer/triglyceride ratios is shown

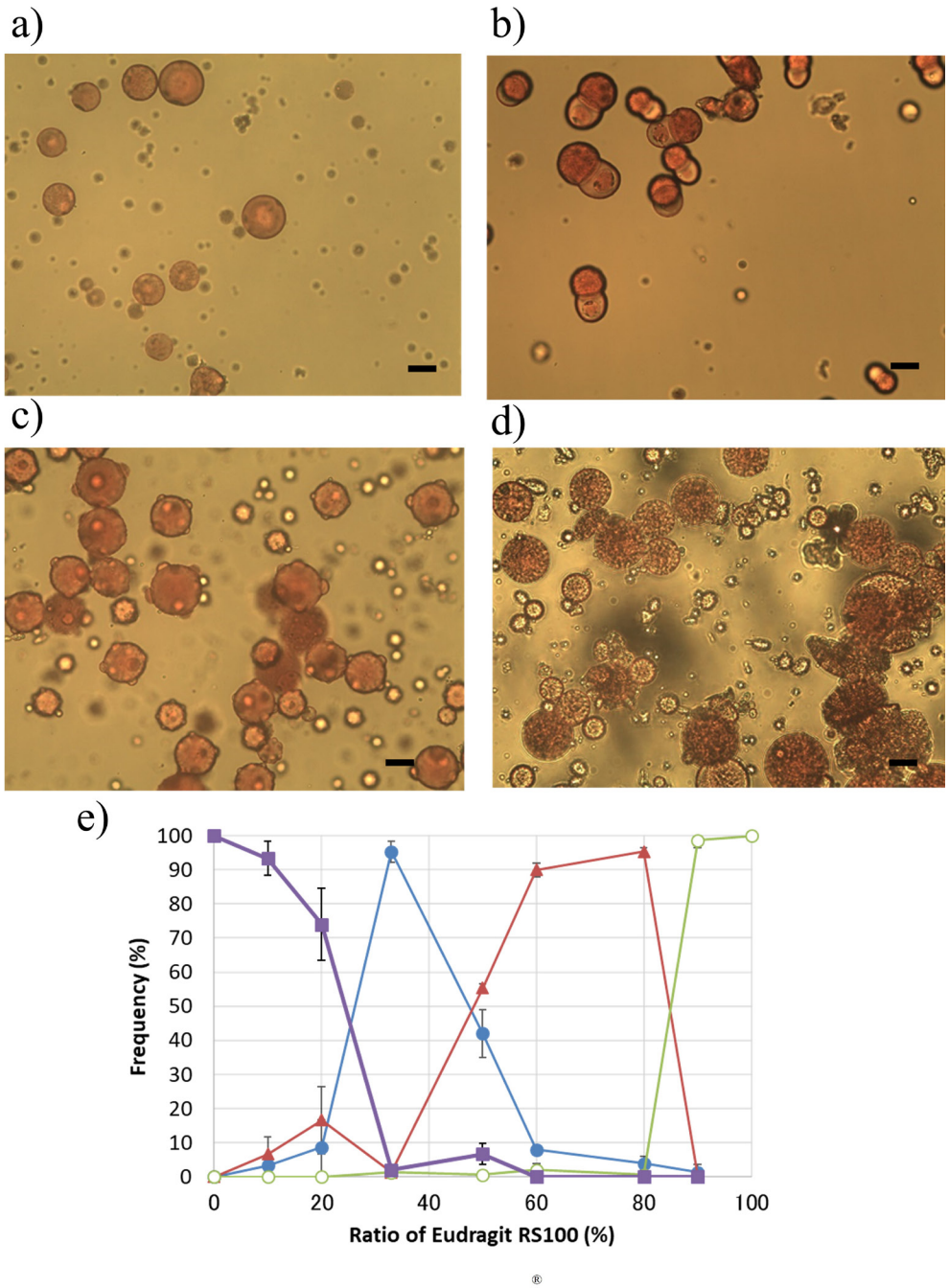

Figure 3. Effect of ammonioalkyl methacrylate copolymer-triglyceride ratio on the conformation of microspheres. Typical images of the optical micrographs of microspheres prepared from **(a)** triglycerides alone, or with a ammonioalkyl methacrylate copolymer and triglycerides, **(b)** 1:2, **(c)** 2:1, and **(d)** 9:1. Bars represent 20 µm. **(e)** Frequency of each conformation depending on ammonioalkyl methacrylate copolymer-triglyceride ratio. Janus microspheres: ● blue line, monolithic microspheres: ○ green line, double-walled microspheres: ■ purple line, star-shaped microspheres: ▲ red line. Data represent mean ± S.D. (n = 3).

in Figure 3e. Janus microspheres were obtained at ammonioalkyl methacrylate copolymer /triglyceride ratios of 1:2 to 1:1, with the maximum yield of these particles [mean ± standard deviation (SD): 90.7% ± 3.1%] being obtained at a ratio of 1:2.

3.3. *DSC analysis of Janus microspheres*

The DSC chart presented in Figure 4 shows the melting points and glass transition temperature of Janus microspheres. Compared with microspheres obtained from ammonioalkyl methacrylate copolymer ammonioalkyl methacrylate copolymer or triglycerides alone, endotherm peaks of Janus microspheres at lower and higher temperatures were identified as the melting of triglycerides and glass transition of the ammonioalkyl methacrylate copolymer, respectively. The endotherm peaks corresponding to the melting of triglycerides in Janus microspheres shifted to a higher temperature than those of triglycerides alone. In contrast, the endotherm peaks corresponding to the glass transition of ammonioalkyl methacrylate copolymer shifted to a lower temperature than those of the ammonioalkyl methacrylate copolymer alone.

3.4. *Distribution of FD4 in Janus microspheres*

Janus microspheres containing FD4 were fabricated using 1% HCO60 as an inner water phase, which did not substantially affect their conformation. The inner water phase of the w/o emulsion during Janus particle formation was finely dispersed. The prepared Janus microspheres incorporated FD4, and optical and fluorescence microscopic observations revealed that the FD4 was localized in the hemisphere containing the ammonioalkyl methacrylate copolymer opposite to that stained by Oil Red O (Figures 5a and 5b). The mean particle size was 31.1 ± 0.5 µm.

3.5. *Dissolution profile of FD4 from Janus microspheres*

The *in vitro* release of FD4 from Janus microspheres was slow, reaching approximately 60% after 5 days (Figure 5c). The cumulative amount of FD4 released from the Janus microspheres obtained was proportional to the 0.75 power of time during a period of 0 to 2 days (correlation coefficient: 0.9984).

3.6. *Observation of Janus microspheres on Caco-2 cell monolayers*

We prepared Janus microspheres loaded with FD4, a hydrophilic fluorescent dye, and Nile red, a hydrophobic fluorescent dye. Nile red and FD4 were observed to be localized in the hard-fat hemisphere and the polymeric hemisphere of the Janus microspheres, respectively. Fluorescence of the middle part of the Janus microspheres became yellow, which is considered to be indicative of interference between the two fluorescent dyes. Therefore, identification of each hemisphere was based on the luminescent color of the tip of each hemisphere, where the tips of ammonioalkyl methacrylate copolymer and hard-fat hemispheres were green due to FD4 and red due to Nile red, respectively.

On addition of Janus microspheres to the apical

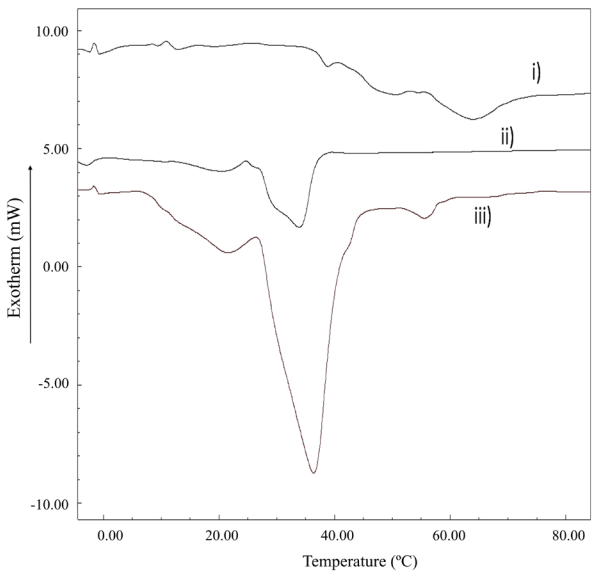

Figure 4. Differential scanning calorimetry chart of Janus microspheres. (i) Ammonioalkyl methacrylate copolymer, **(ii)** Triglycerides, **(iii)** Janus microspheres (ammonioalkyl methacrylate copolymer/triglycerides = 1/2).

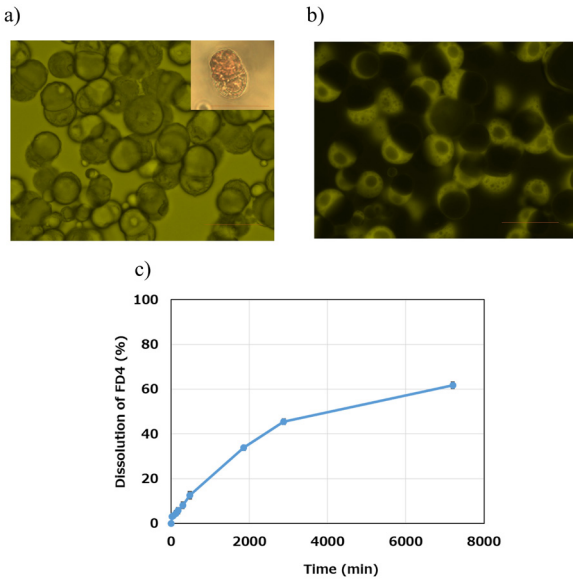

Figure 5. Distribution of fluorescein isothiocyanate-dextran (FD4) in Janus microspheres. (a) Optical micrograph image and **(b)** fluorescence micrograph image. **(c)** FD4 release profiles from Janus microspheres. The dissolution test was performed at 37C using a second fluid (pH 6.8) containing 0.05% Tween 80. Bars represent 50 µm.

side of a Caco-2 monolayer, we observed that most of the particles settled onto the surface of the monolayer within 5 min, with the polymeric hemisphere oriented downwards and the hard-fat hemisphere facing upwards (Figures 6a and 6b). We detected no Janus microspheres with hard-fat hemisphere attachment. Side-on attachment was rarely observed, whereas in contrast, 22.3 ± 3.4% of the precipitated particles were found to be side-on at the bottom of wells lacking a monolayer. After overnight incubation, 6.01 ± 0.79 × 10^3 particles/cm^2 of the Janus microspheres were observed on the surface of the Caco-2 monolayer. In addition, even after washing with the PBS(-), we observed that 42% of the particles (2.53 ± 0.48 × 10^3 particles/cm^2) remained attached to the monolayer (Figures 6c and 6d).

4. Discussion

The phase separation method is based on a phenomenon in which phase separation occurs between two materials as the solvent is evaporated. We have previously reported

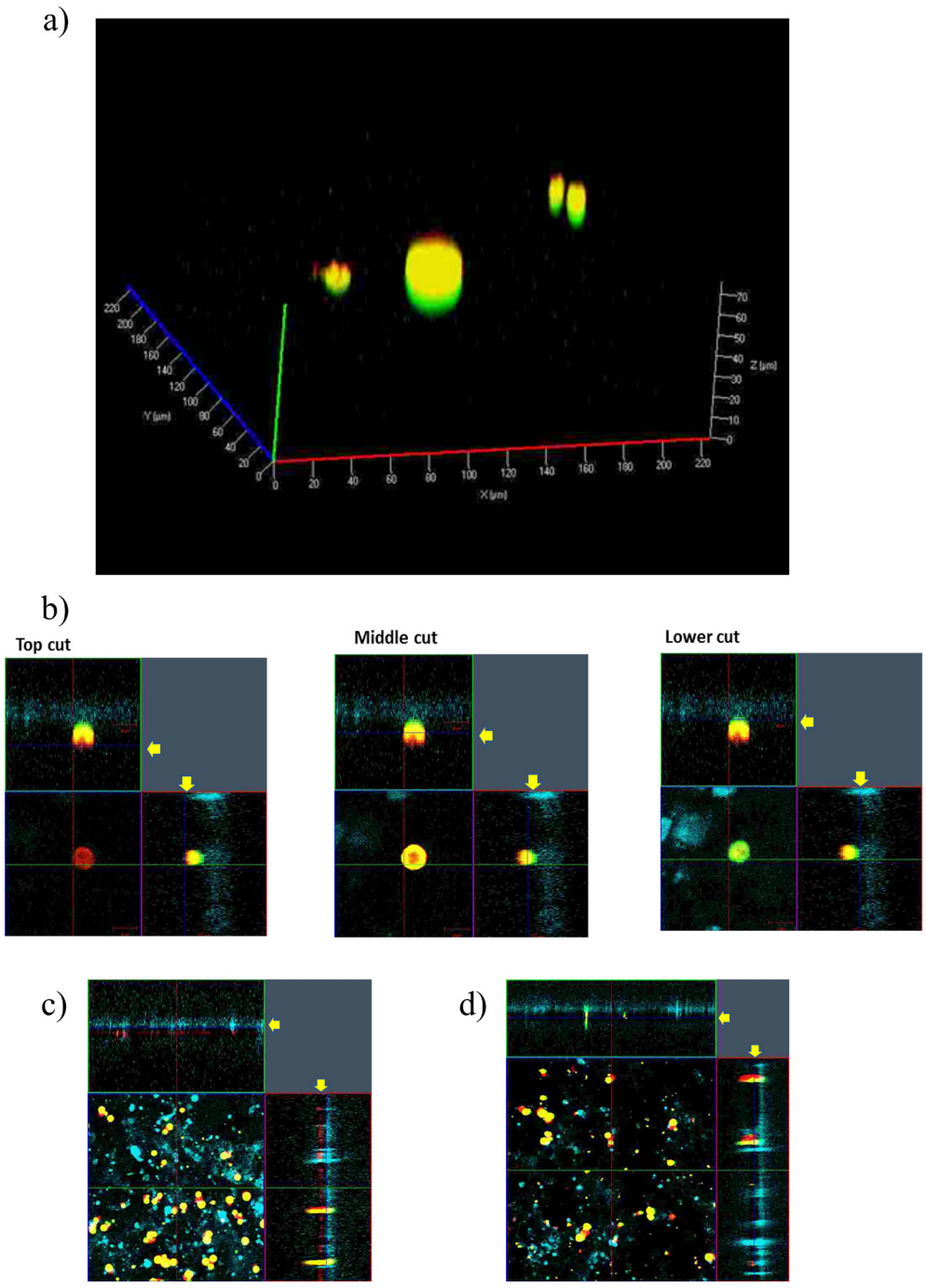

Figure 6. Confocal images of Janus microspheres on a Caco-2 monolayer. Images of Janus microspheres after 30 min incubation, **(a)** on an unstained Caco-2 monolayer and **(b)** on a cell tracker violet-stained Caco-2 monolayer. Images of Janus microspheres after overnight incubation, **(c)** before and **(d)** after washing.

that solvent evaporation following formation of an o/w-type emulsion can yield Janus microspheres composed of poly (lactide-co-glycolide) and triglycerides (*28*). Phase separation between poly (lactide-co-glycolide) and triglycerides occurs in a dispersed oil droplet as the solvent is evaporated. In addition, we revealed that the decremental profiles of solvent in an oil phase influences the stability of the conformation as Janus microspheres. In the present study, we applied optimized conditions based on conformation stability, as reported in a previous study, for the fabrication conditions of Janus microspheres composed of ammonioalkyl methacrylate copolymer and triglycerides. Initially, we examined whether phase separation occurred in an ammonioalkyl methacrylate copolymer, triglyceride, and methylene chloride ternary system. Although methylene chloride is a good solvent for both ammonioalkyl methacrylate copolymer and triglycerides, phase separation was observed above a certain concentration, referred to as the critical concentration. The critical concentration between ammonioalkyl methacrylate copolymer and triglycerides was found to be higher than that between poly (lactide-co-glycolide) and triglycerides in methylene chloride, as reported previously (*28*). In addition, at the critical concentration of the ammonioalkyl methacrylate copolymer/triglyceride/methylene chloride ternary system, the solution showed higher viscosity than that of the poly (lactide-co-glycolide)/triglyceride/methylene chloride ternary system. This is not only due to the higher critical concentration of the ammonioalkyl methacrylate copolymer/triglyceride/methylene chloride ternary system but also because the ammonioalkyl methacrylate copolymer (molecular weight: 32 kDa) used in this study has a higher molecular weight than the poly (lactide-co-glycolide) used in the previous study (molecular weight: 20 kDa) (*28*). The higher viscosity is a notable point. For mixtures of ammonioalkyl methacrylate copolymer and triglycerides, phase separation in the dispersed oil phase is believed to occur at higher viscosity than that for mixtures of poly (lactide-co-glycolide) and triglycerides in the process of solvent evaporation as reflected in the results of phase diagram analysis. In our previous study, we proposed a mechanism whereby Janus microspheres are formed: the droplets produced by phase separation in the dispersed oil phase of the emulsion aggregate and coalesce before the coalescing droplets migrate to form two layers, resulting in the formation of Janus microspheres (*28*). It is believed that obtaining a Janus conformation using mixtures of ammonioalkyl methacrylate copolymer and triglycerides is more difficult because high viscosity may prevent coalescence and migration of the separated droplets. Our data revealed that the formation of Janus microspheres is influenced by the ratio of ammonioalkyl methacrylate copolymer to triglycerides. A high ratio of ammonioalkyl methacrylate copolymer (50-80%) yielded star-shaped microspheres, but not Janus microspheres, and resulted in a higher viscosity at the critical concentration than lower ratios. The development of microspheres with a star-shaped conformation may be attributable to the fact that although the limited droplets separated in the oil phase can migrate to the surface of the oil phase, the droplets will be prevented from coalescing due to the high viscosity in the oil phase. Moreover, we also found that the size of droplets on the surface of a microsphere decreased as the ratio of ammonioalkyl methacrylate copolymer to triglycerides increased (*i.e.*, as the viscosity of the oil phase increased). However, increasing the proportion of ammonioalkyl methacrylate copolymer to more than over 90% yielded monolithic microspheres. This is believed to be attributable to the formation of a solid solution of triglycerides and ammonioalkyl methacrylate copolymer. Decreasing the glass transition temperature, as determined from the DSC chart, indicated that a certain amount of triglyceride had dissolved in the ammonioalkyl methacrylate copolymer.

Notably, a high ratio of triglycerides yielded double-walled microspheres, similar to the microspheres prepared from triglycerides alone. The triglycerides used in this study were mixtures of triglycerides containing various acyl chains, and the double-wall conformation is believed to be a consequence of phase separation between the different triglycerides. When microspheres were prepared with 80% triglycerides, double-walled microspheres were also obtained. However, we were not able to clarify the origin of the droplets in these microspheres, *i.e.*, whether they were derived from triglyceride components or separation of the ammonioalkyl methacrylate copolymer phase.

Janus microspheres were found to be obtained within a narrow range of the ammonioalkyl methacrylate copolymer/triglyceride ratios (between 1:2 and 1:1), at which the viscosity of the dispersed oil phase permitted the separated droplets to migrate to the surface and coalesce to form hemispheres during phase separation. The Janus microspheres thus contained triglyceride droplets within the ammonioalkyl methacrylate copolymer hemisphere, which are believed to be formed from glycerides with short acyl chains. The concentrations at which a phase separation between triglyceride and ammonioalkyl methacrylate copolymer occurred decreased as increasing the length of the acyl chains of triglycerides: Suppocire AM (C12-C18) < tripalmitate glyceride (C16) < Triester F810 (C6 and C8). This indicated that the phase separation of short-chain triglycerides in the dispersed oil phase occurred later in the solvent evaporation process than that of long-chain triglycerides. The separated droplets of long-chain triglycerides can migrate to the surface of the dispersed oil droplets and coalesce, as described above. However, when droplets of short-chain triglycerides were separated, the viscosity of the dispersed oil phase was too high for the droplets to migrate to the surface.

We prepared Janus microspheres loaded with FD4

using the w/o/w-type emulsion solvent evaporation method with an inner water phase of 1% HCO60. The triglyceride hemisphere of the Janus microspheres was stained with Oil Red O, as previously reported (*28*), and the fluorescence of FD4 was detected in the other (polymeric) hemisphere. When phase separation between the polymer and triglycerides occurred in the oil phase, the inner water phase containing FD4 was distributed in accordance with the partition phenomenon. Because the ammonioalkyl methacrylate copolymer is more hydrophilic than triglycerides, the inner water phase should tend to be in ammonioalkyl methacrylate copolymer hemispheres.

The cumulative dissolution rate of FD4 from the Janus microspheres was proportional to the 0.75 power of time (correlation coefficient: 0.9984) over a period of 0-2 days. Among mathematical models used for dissolution from pharmaceutical formulations, the Higuchi model is known to show release proportional to the square root of time (*30*). This model can be applied to an erodible formulation with drug homogeneously dispersed in a matrix; however, the model does not consider swelling and interaction between polymer and drug. In the case of Janus microspheres composed of an ammonioalkyl methacrylate copolymer and triglycerides, drug release from the ammonioalkyl methacrylate copolymer hemisphere is also speculated to be controlled by swelling of the polymer and electric interaction between the polymer and FD4. Namely, ammonioalkyl methacrylate copolymer has quaternary ammonium groups, which show cationic charges under acidic and neutral conditions. The exchange of the quaternary ammonium group counterions with the surrounding medium develops a water flux within which drug molecules can diffuse out of the dosage form (*31*). The attraction of ions in the release medium to the quaternary ammonium groups determines the extent of water flux and hence the drug release rate (*32*). In addition, the contribution of the interaction between ammonioalkyl methacrylate copolymer and FD4, which are cationic and anionic, respectively, at pH 6.8 in the dissolution test, has to be taken into consideration. Drug release through the interaction *via* electric charge is known to be related to ion exchange, and ion exchange with the chloride ion is considered to influence the permeability and drug release of Eudragit RS30D films (*33*). Therefore, the swelling and ionic interaction as an additional release mechanism may be one reason why release from the Janus microspheres differs from that predicted by the Higuchi model.

The direction of release of a hydrophilic drug from Janus microspheres will be limited to the side of the polymeric hemisphere because the triglyceride hemisphere functions as an impermeable backing. The polymeric hemisphere has two surfaces, *i.e.*, the spherical and flat surfaces, with the latter lying at the interface with the triglyceride hemisphere. We speculate that hydrophilic drugs are released predominantly from the spherical surface compared with that from the flat surface. At polymer interfaces, polymers have been shown to be orientated in hydrophilic or hydrophobic groups along an adjacent material depending on the properties of the adjacent material (*34*). The ammonioalkyl methacrylate copolymer has both hydrophilic and hydrophobic groups, and at the interface of an ammonioalkyl methacrylate copolymer hemisphere with a triglyceride hemisphere, the ammonioalkyl methacrylate copolymer is believed to form a hydrophobic layer composed of its hydrophobic moieties along the adjacent triglycerides (hydrophobic materials). Hence, the interface between the ammonioalkyl methacrylate copolymer hemisphere and the triglyceride hemisphere may be so hydrophobic that it restricts the access of water to the interface, thereby inhibiting the release of a hydrophilic drug. In contrast, the surface of the spherical part of the hemisphere is covered by the hydrophilic group of the ammonioalkyl methacrylate copolymer because the surface comes in contact with water during fabrication. Accordingly, water can gain access to the spherical part of an ammonioalkyl methacrylate copolymer hemisphere, thereby facilitating release of the incorporated hydrophilic drug.

Our investigation using Caco-2 monolayers revealed that the Janus microspheres can attach onto the monolayer in an orientated manner. Only a few agglomerates were observed in the present study. With regards to the particles existing as a primary particle, the attachment to the monolayer can be categorized into four groups, namely, (a) unattached, (b) polymeric hemisphere-oriented attachment (c) hard-fat hemisphere-oriented attachment, and (d) side-oriented or laterally oriented attachment. In this study, most of the Janus microspheres were observed to be attached to the apical surface of the monolayer *via* the ammonioalkyl methacrylate hemisphere. No particles with hard-fat hemisphere-oriented attachment were detected, and only a few particles with side-oriented or laterally oriented attachment were observed, which was characterized by polymeric and hard-fat spheres combined to form a shape resembling a figure eight (data not shown). The orientation of Janus microspheres on the Caco-2 monolayer may be due to difference in the specific gravity of the two hemispheres. This is supported by data showing that 78% of the Janus microspheres were observed in polymeric hemisphere-on settlement on the walls of culture plate wells lacking cells. The specific gravity of the polymeric hemisphere is higher than that of the hard-fat hemisphere, and we found that 42% of the particles remained attached to the monolayer after overnight incubation. We thus believe that the polymeric hemisphere of Janus microspheres may initially attach to Caco-2 monolayers *via* gravity, followed by strong binding to the surface. The major binding force is considered to be electrostatic. Although the surface of the Caco-2 monolayer appears to be

hydrophobic, it is known that charged particles can become strongly attached. In studies using liposomes (*35*) and oil droplets (*36*), it has been demonstrated that cationic particles can attach to Caco-2 monolayers to a greater extent than anionic and neutral particles. The polymeric hemisphere-orientated attachment of the Janus microspheres to Caco-2 monolayers observed in the present study appears to be consistent with the findings of these studies, because these microspheres consist of cationic polymer and neutral hard-fat hemispheres. Thus, the oriented attachment of Janus microspheres to the monolayer appears to be attributable mainly to the electrostatic properties of the two hemispheres, although the contribution of gravity remains to be fully clarified.

Mucin is a highly glycosylated protein with oligosaccharide side chains containing terminal sialic acid and sulfate residues (*37,38*), the negative charges of which can interact with cationic particles. The non-glycosylated regions of the mucin protein backbone and lipids associated with mucin contribute to the hydrophobicity of this protein, which can interact with hydrophobic materials. These properties of mucin may influence the orientation of Janus microspheres *in vivo*. Positively charged oil droplets have been reported to exhibit stronger binding to mucosal surfaces than negatively charged droplets (*39*) and also to bind to the surface of Caco-2 cells by electrostatic attraction (*36*). Taking these details into consideration, it appears that the oriented attachment on a mucosal surface is similar to that on Caco-2 cells, which do not secrete mucin.

In order to achieve focused drug release in a specific direction, we have recently demonstrated that the condensation of insulin within a limited surface area or the dimple of a hard-fat suppository, which releases a high concentration of insulin only toward the mucosal side within a limited area, reduces blood glucose levels following rectal administration (*40*). This supports our concept shown in Figure 1. We are currently performing experiments to obtain proof of concept.

Particle size is an important factor affecting intestinal drug absorption *via* a particulate delivery system, and in the present study we adjusted the size of the particles to approximately 30 μm in order to observe the disposition of a drug contained within Janus microspheres. As particles on the nanometer scale can readily penetrate the mucus layer to access the epithelial surface (*41*), nanosized Janus particles may function as a patch, adhering directly to the apical membranes of enterocytes. Accordingly, the feasibility of reducing the size of Janus microspheres should be evaluated in further studies.

In conclusion, in the present study, we demonstrated that Janus particles prepared using an ammonioalkyl methacrylate copolymer and a hard fat could be fabricated using the w/o/w emulsion solvent evaporation method to release a model hydrophilic drug from a single hemisphere. These mucoadhesive Janus microspheres may be useful for the non-parenteral, particularly enteral, mucosal delivery of biomedicines. In future studies, the adhesion to mucous membranes and local retainability of Janus microspheres should be further investigated *in vivo* under conditions relevant to drug delivery.

Acknowledgements

We would like to thank Ms. Kayoko Murakami for her technical assistance. We are also grateful to Editage (www.editage.jp) for English language editing. We gratefully acknowledge the financial support for this study provided by CBC Co., Ltd.

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(Received November 30, 2019; Revised December 20, 2019; Accepted December 21, 2019)