Formulation of Uniform-sized Agar Gel Microbeads from Water–in–oil Emulsion Prepared Using Microchannel Emulsification under Controlled Temperature

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Size–controlled preparation of agar gel microbeads using monodisperse water–in–oil (W/O) emulsion was investigated. W/O emulsions were prepared by microchannel (MC) emulsification using three grooved MC plates with different geometries of the MC region. The addition of sodium chloride to the dispersed phase was necessary for stable preparation of monodisperse agar-containing W/O emulsions. The mean droplet size varied from 15 to 34 μm depending on MC geometry, while the coefficient of variation was below 10%. Monodisperse agar gel microbeads were obtained by cooling the emulsion droplets prepared at a temperature higher than the gel point of the agar solution. Emulsification conditions (e.g., agar concentration and emulsification temperature) affected droplet diameter and uniformity of the W/O emulsions. Monodisperse and quasi-monodisperse W/O emulsions could be prepared at agar concentrations of 0.5 to 2.0 wt% and at emulsification temperatures of 40 to 50 ℃, which exceeded the gel point of the agar solution. At temperatures below the gel point, MCs were partly clogged by the to-be-dispersed agar solution, due to partial gelation of the agar solution. However, above 50 ℃, emulsification was destabilized because of immediate coalescence of droplets formed once downstream of MCs.

Keywords: microchannel emulsification, agar gel, water–in–oil emulsion, size control, gel point

1. Introduction

Agar is a polysaccharide extracted from seaweed (e.g., Gelidium, Gracilaria, Pterocladia, and Ahnfeltia) and consists of agarose (70%) and agarpectin (30%) [1]. It is insoluble in cold water but soluble in hot water. Agar solution (1 wt%) forms a gel via formation of intramolecular and intermolecular hydrogen bonds when it is cooled to 30 to 40 ℃. Agar gels are widely used as foods and as a culture medium for microorganisms.

Agar and agarose gel beads can be used as encapsulation carriers for nutrients, drugs, and microorganism cells, and as a separation matrix in column chromatography. In the preparation of agar gel beads, it is important to control bead size. Gel bead size affects the diffusive transportation of nutrients and oxygen from the external medium, release properties of encapsulated compounds to the external phase, and separation properties for gel chromatography [2]. Smaller uniform-sized agar beads should offer many advantages, and better mechanical strength and better dispersion property are expected for small beads.

Preparation of agar or agarose gel beads is based mainly on water–in–oil (W/O) emulsions that consist of agar or agarose solution as the dispersed aqueous phase and water-immiscible organic solvents including hydrocarbons and natural oils as the continuous phase. The dropping–cooking method [3] is quite simple and does not need any special apparatus for preparing beads 800 to 1000 μm in mean diameter; however, preparation of beads of less than 300 μm with narrow size distribution has been impractical to date. A widely applied method of preparing agar and agarose-containing W/O emulsion is emulsification by agitation. With this method, agar and agarose gel beads from submicrometer to several tens of micrometers may be obtained using a homogenizer at 50 to 80 ℃ and typically 5000 to 24000 rpm rotator speed [4, 5]. With this method, relatively small particles can be easily obtained. However, their size distribution is usu-
ally polydisperse, and unstable encapsulated components (e.g., bioactive proteins and cells) are inactivated due to generation of shear force and heat. During the past decade, microfluidic approaches have attracted much attention as novel methods for precisely preparing polysaccharide hydrogels. Using flow–focusing junction [6], parallel–flow double–nozzle [7, 8], and micronozzle array [9] devices, polysaccharide hydrogels including agarose gel beads with 80 to 300 μm mean diameter and narrow size distributions were successfully obtained. With these methods, droplet size is determined using the balance between interfacial force and drag force by viscous flow. Because the flows in these microfluidic devices are laminar due to the low Reynolds number (10^3 usually) [10], they are often difficult to creating small droplets (less than 50 μm) with high productivity.

An alternate approach is droplet formation driven by interfacial tension using uniform, small pores or precisely fabricated channels through which a polysaccharide solution passes and spontaneously transforms into droplets. This method’s advantages include unique droplet generation via a low–shear process [11], expected suitability for encapsulating labile components such as heat– or shear–sensitive proteins and cells. Zhou et al. [12] reported the preparation of agarose gel beads from W/O emulsions produced by membrane emulsification at 55°C using a microporous glass membrane. In their study, uniform–sized agarose beads with a mean diameter of 15 to 60 μm and a coefficient of variation (CV) below 15% were obtained. In previous studies [13], the authors demonstrated a novel method of preparing uniform–sized alginate microparticles of several micrometers in diameter using the combination method of microchannel (MC) emulsification [14, 15] and external gelation. Using MC emulsification, alginate–containing droplets with a mean diameter of 20 μm and a CV below 10% could be obtained. In MC emulsification, droplets are generated spontaneously via an interfacial tension–driven process [16] without generating severe shear force and heat. In recent works, the productivity of emulsion droplets has been remarkably improved using high–density integration of MCs in a single chip (> 10^4 – 10^5 channels per chip) [11, 17, 18].

In this study, we focus on preparing agar gel microbeads of less than 50 μm using MC emulsification. Since agar is considerably cheaper than agarose, the preparation of agar gel microbeads has a potential impact for scientific and industrial applications. To prepare agar–containing W/O emulsions, the temperature must be kept above the gel point temperature of agar solutions to prevent gelation during emulsification. Details of the effects of temperature on MC emulsification have been reported only recently [19–21]. MC emulsification at temperatures above room temperature was reported in some cases when gelatin [22, 23], alginate [13], and tripalmitin [24] were used as dispersed phase components. Examining the temperature effect on MC emulsification using agar solution should be informative from both fundamental and application viewpoints.

2. Materials and methods

2.1 Chemicals

Agar powder; Span 85 (sorbitan trioleate); 1, 1, 1, 3, 3–hexamethyldisilazane (HMDS); and sodium chloride (NaCl) were purchased from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). Isocetane (2, 2, 4–trimethylpentane) was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). The water used in all experiments was prepared using a Direct–Q water purified system (Merck Millipore Corporation, Billerica, USA) and had 18.2 MΩ cm resistivity.

2.2 Preparation of Agar Microbeads Using MC Emulsification

Monodisperse W/O emulsions were prepared by MC emulsification using laboratory–scale MC emulsification modules with grooved silicon MC plates. By using the grooved–type MC plates, emulsification behavior at the MC region can be observed directly via a microscope video system as described below. In this study, three MC plates (one cross–flow (MC–CF) and two dead–end (MC–DS and MC–DL) plates) with different dimensions [16, 25] (MC depth (D_Mc), MC width (W_Mc), and terrace length (L_t)) were used (Figs. 1a, b). To prepare W/O emulsions, the silicon MC plates and the glass plates were treated with HMDS to obtain hydrophobic MCs as described previously [13].

Agar powders were suspended in water containing 0 or 0.2 M NaCl in a test tube by mixing using a vortex mixer. The mixtures were heated at 95°C in a dry block bath (Model MG–2200, EYELA, Tokyo, Japan), and the resulting clear solution was used as a to–be–dispersed phase during emulsification. The to–be–dispersed phase was introduced into the module via flexible tubing connected to a syringe installed in a syringe pump (Model SPE–1, AS ONE Corporation, Osaka, Japan). The flow rate of the dispersed phase was set to 0.2 mL/h unless otherwise
stated.

Isooctane containing 3 wt% Span 85 was prepared; then, the isooctane solution was saturated with water by letting the solution to be in contact with water at a volume ratio of 9:1 (isooctane solution: water) for 30 min, followed by centrifugation (2500 rpm for 15 min with a table centrifuge) to obtain phase separation. The supernatant isooctane solution was recovered for use as the continuous phase. This continuous phase was introduced into the MC module via flexible tubing connected to a syringe installed in the syringe pump. The flow rate of the continuous phase was fixed to 2.9 mL/h in all experiments.

The to-be-dispersed phase was forced through the MCs into the continuous phase to generate emulsion droplets (Fig. 1c). During emulsification, the MC module and the syringe containing the agar solution were heated using a ribbon heater equipped with a voltage converter to control temperature. Emulsification was observed using a color CCD camera (WAT-231S2, Watec, Tokyo, Japan) or a digital camera with a high-speed mode (Nikon J1, Nikon, Tokyo, Japan) attached to a micro-

![Diagram](image-url)

Fig. 1 Schematic illustrations of MC plates used in this study. (a) Plate with MC-CF (cross-flow). (b) Plate with MC-DS and -DL (dead-end). (c) Schematic illustration of droplet formation using the MC plates.
The prepared W/O emulsion in the MC module was collected by flowing additional continuous phase through the module.

2.3 Analytical methods

The diameters of the emulsion droplets and microbeads were determined by measuring the diameter of the captured images of at least 100 droplets or beads using Microsoft PowerPoint software. CV was calculated based on the following equation:

\[ CV = \frac{\sigma}{d_m} \times 100 \]

where \( \sigma \) is the standard deviation and \( d_m \) is the mean diameter.

Viscosities of both the dispersed phase and the continuous phase were measured using a vibrational viscometer (SV-10, A&D, Tokyo, Japan) equipped with a sample chamber with a water circulator to control sample temperature.

Interfacial tension between the agar solution (1 wt%) and the continuous phase was determined using the pendant drop method. The profile of a drop of agar solution suspended in isooctane containing 3 wt% Span 85 was determined using an automatic interfacial tensiometer (DM-301, Kyowa Interface Science Co., Ltd., Niiza, Japan). Each measurement was repeated at least 20 times, and the calculated mean values were used.

3. Results and discussion

3.1 Effect of NaCl addition to the dispersed phase

W/O emulsion droplets were prepared as precursors for formulating uniform-sized agar gel microbeads. In this study, grooved MC plates, on which highly uniform microgrooves with a slit-like terrace and a deeply etched well [16] were fabricated, were used for emulsification experiments. First, the effect of NaCl addition on the production of W/O emulsions was investigated using an MC-CF plate. Figure 2 illustrates typical emulsification behaviors using 1 wt% agar solution with and without the addition of 0.2 M NaCl at 45°C. Polydisperse W/O emulsions consisting of large and small droplets were produced from the outlets of MCs without NaCl in the dispersed phase. In this case, the terrace was partially wetted by the agar solution, and the dispersed phase continuously flowed out to the continuous phase; thus, polydisperse droplets formed (Fig. 2a). In contrast, highly uniform droplets containing agar were generated from the outlets of MCs when 0.2 M NaCl was added to the to-be-dispersed agar solution (Fig. 2b). The resultant agar-containing droplets had narrow size distributions with \( d_m \) of 23 μm and CV of 7%, demonstrating that monodisperse W/O emulsions were produced. As demonstrated in previous studies [26], the osmotic pressure of the to-be-dispersed phase should affect MC emulsification behavior. Kobayashi et al. [26] reported that osmotic pressure of at least 0.42 MPa was necessary to produce monodisperse aqueous droplets. The osmotic pressure of 0.2 M NaCl solution was calculated as 1.1 MPa using the van’t Hoff equation [27], \( \Pi = iMRT \), where \( i \) is the van’t Hoff factor with a value of 2 for NaCl, \( M \) is the molar concentration of NaCl [mol m\(^{-3}\)], \( R \) is a gas constant with a value of 8.31 Pa m\(^3\) K\(^{-1}\) mol\(^{-1}\), and \( T \) is the thermodynamic temperature [K]. This osmotic pressure was sufficient to produce monodisperse W/O emulsions; thus, we added 0.2 M NaCl to the agar solution in the following investigations.

As depicted in Fig. 3, the mean diameter of agar-containing droplets or agar gel microbeads was 15.1 μm obtained using MC-CF, 23.4 μm using –DS, and 33.8 μm using –DL; the CV values were within 4 to 7% in each case. There was no difference between the diameter of
droplets/microbeads with different mean droplet diameters can be produced using MC emulsification. The obtained droplet diameters were roughly 3 times larger than each MC’s depth, although the actual droplet diameter would be affected by other parameters of the MC geometry, such as terrace length [28]. The results also confirm that the mean diameter depends on the geometry of the MC. They also suggest that we can produce agar-containing aqueous droplets with various mean diameters using MC plates with appropriate dimensions (the minimum droplet diameter generated by currently-available MC plate is about 1 μm [11]), even though the droplet diameter is affected by both the dimensions of the MCs and the physical properties of experiment components, including the viscosities of both dispersed and continuous phases [26, 29-31]. In later sections, we use MC-DS for emulsification experiments.

3.2 Droplet formation behavior

Figure 4 presents successive photomicrographs of typical droplet formation behavior at the end part of MC-DS taken using the high-speed mode (1250 frames/s) of a digital camera equipped with the microscope system in the MC experiment setup. Agar concentration in the to-be-dispersed phase was 1 wt%, and emulsification temperature was 47°C. In this experiment, the flow rate of the to-be-dispersed phase was set to 0.04 mL/h for clear observation of the movement of the liquid-liquid interface. Droplet formation at the MC through which the to-be-dispersed phase passed was similar to that reported previously [16]. To-be-dispersed agar solution passed through the MC inflated on the terrace in a disc-like shape in 0.4 s (inflation time). When the agar solution reached the end of the terrace, it flowed into the deep well and transformed into a spherical shape in 0.01 s (detachment time). This transformation-detachment process was driven by interfacial tension. Indeed, in this experiment, a few droplets to a couple of dozen droplets...
were generated per second from each MC, varying widely for individual MCs. This result indicates that the time needed for generating one droplet varied from 0.05 s to 0.5 s. This time difference was due mainly to variation in inflation time. The difference in detachment time was not obvious among different MCs. The interfacial shape of the to-be-dispersed phase on the terrace was symmetric to the center line of the MC throughout the entire droplet generation, indicating that the attractive force did not act between the hydrophobized MC/glass plates and dispersed phase stabilized by the non-ionic surfactant Span 85 [21]. Using isooctane containing 3 wt% Span 85, agar-containing droplets could be produced smoothly and uniformly in MC emulsification.

3.3 Effect of agar concentration

Figure 5 depicts the effect of agar concentration of the to-be-dispersed phase on the droplet diameter of agar-containing W/O emulsions. The emulsification temperature was controlled at 45.5±2°C. When the agar concentration increased from 0.5 wt% to 1.0 wt%, the mean droplet diameter decreased from 18.8 μm to 17.2 μm, and CV decreased from 13% to 7%. However, increases in mean droplet diameter and CV were observed with a further increase of agar concentration from 1.0 wt% to 2.0 wt%. The viscosity of agar solution increased with increased agar concentration (2.9 to 12.6 mPa s at 45°C). The viscosity of the continuous phase was 0.43 mPa s at 45°C. In previous studies [26, 29, 30], droplet diameter in W/O emulsions prepared using MC emulsification decreased with increased ratio of viscosity of the dispersed phase to that of the continuous phase. In this study, the same tendency was obtained at low agar concentrations (0.5 to 1.0 wt%). However, at higher agar concentrations (2.0 wt%), mean droplet diameter increased despite the increase in viscosity ratio. In fact, at 2.0 wt% concentration, droplet diameter became smaller (15 μm); however, obviously large droplets (over 20 μm) also formed at the same time (Fig. 5, inset pictures). Droplet diameter distributions also indicated this situation (data not shown). Formation of smaller droplets at 2 wt% agar concentration should be due to the increase of the viscosity ratio. However, larger droplet formation might be caused by the instability of emulsification. In this experiment, the emulsification temperature was kept at 45.5±2°C, which is very close to the gel point of 2 wt% agar solution (44°C). At this agar concentration, partial adhesion and wetting [32] by the to-be-dispersed phase was caused by gelation and increased viscosity of the to-be-dispersed phase containing 2 wt% agar, resulting in unstable formation of larger droplets. These results indicate that the temperature during MC emulsification using agar solution is important and affects droplet diameter and uniformity.

These experiments confirmed that monodisperse (CV<10%) or quasi-monodisperse (CV<15%) [15, 17] agar-containing aqueous droplets could be successfully prepared at all agar concentrations ranging from 0.5 wt% to 2.0 wt% using MC emulsification, although their size uniformity depended on the experiment parameters.

3.4 Effect of emulsification temperature

Figure 6 plots the effect of emulsification temperature on droplet diameter and uniformity. At 39°C, monodisperse droplets ($d_m=15.1\ \mu m$, CV=4%) were obtained, although part of the MC was clogged by the dispersed phase. The gel point of 1 wt% agar solution was determined to be 41°C, and the viscosity of 1 wt% agar solution was drastically increased below 40°C (Fig. 7). Therefore, emulsification temperature above the gel point of agar solution should be suitable for stable preparation of W/O emulsions. In fact, for emulsification temperatures between 40 and 50°C, monodisperse W/O emulsions with CV of less than 10% were successfully obtained. With temperatures above 50°C, polydisperse W/O emulsions were obtained mainly due to quick coalescence after droplet formation. Previous studies reported stable preparation of W/O emulsions using MC emulsification at...
emulsification temperatures between 10 and 55°C [21]. In this study, equilibrium interfacial tension decreased with increased temperature, being similar to previous data for nonionic surfactants [19–21]. Therefore, unstable MC emulsification over 50°C in this study might be due to the nature of the agar solution; however, details of its mechanism were not clear. At emulsification temperature above 50°C, large droplet formation via quick coalescence was observed microscopically just after droplet generation downstream of the MC region. However, droplets did not frequently coalesce further downstream in the MC module. Thus, some differences in adsorption of nonionic Span 85 on the liquid–liquid interface at high temperature might be one cause of droplet instability. The emulsifier must be transported diffusively and adsorb to the newly created interface. To stabilize the newly created interface via coverage with the emulsifier, the diffusion and adsorption of emulsifier molecules must be sufficiently fast [19, 32, 33]. Hence, the stability of the newly created interface should be dominated by the relative rate between the diffusion–adsorption process of emulsifier molecules and the creation of the droplet interface. At higher temperatures, the balance of these rates might change by the decrease of the relative adsorption rate. Since the adsorption enthalpy of the nonionic surfactant is generally less than 0, adsorption equilibrium should shift to the desorption side at higher temperatures. This equilibrium shift might decrease the relative adsorption rate. In addition, the hydrophilic head groups of nonionic emulsifier molecules would be dehydrated at a high temperature; hence, their hydrophilicity would decrease. This phenomenon might also affect the adsorption of the emulsifiers as well as the stability of the formed droplets. Furthermore, the kinetics of the rupture of a thin film between two contact droplets induced by thermal fluctuation of the droplet-droplet contact surfaces can be described as a function of temperature: film rupture frequency increases with increased temperature [34]. To clarify the mechanism of variation of emulsification at high temperatures, further investigations must be carried out.

4. Conclusions

Preparation characteristics of agar-containing W/O emulsion for formulating uniform-sized agar microbeads using MC emulsification were demonstrated. Adding NaCl to the dispersed agar solution to provide sufficient osmotic pressure was necessary for stable formation of highly monodisperse droplets. Monodisperse agar-containing W/O emulsions and agar microbeads with a mean diameter of 15 to 34 μm could be prepared using...
various MC plates with different MC geometries. Monodisperse and quasi-monodisperse W/O emulsion droplets could be formed at agar concentrations of 0.5 to 2.0 wt% and emulsification temperatures of 40 to 50°C. Mild, low-shear MC emulsification is expected to be advantageous for preparing both uniform-sized agar microbeads and those containing labile components such as bioactive proteins and cells.

**Nomenclature**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>$D_c$</td>
<td>channel depth, $\mu$m</td>
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<tr>
<td>$d_m$</td>
<td>mean droplet diameter, $\mu$m</td>
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<tr>
<td>$i$</td>
<td>van’t Hoff factor, $-$</td>
</tr>
<tr>
<td>$L_t$</td>
<td>terrace length, $\mu$m</td>
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<tr>
<td>$M$</td>
<td>molar concentration, mol·m$^{-3}$</td>
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<tr>
<td>$R$</td>
<td>gas constant, Pa·m$^3$·K$^{-1}$·mol$^{-1}$</td>
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<tr>
<td>$T$</td>
<td>thermodynamic temperature, K</td>
</tr>
<tr>
<td>$W_c$</td>
<td>channel width, $\mu$m</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>standard deviation of droplet diameter, $\mu$m</td>
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**References**

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温度制御下でのマイクロチャネル乳化により調製した单分散油中水滴エマルションからの均一径寒天ゲルマイクロビーズの作製

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寒天およびその主成分であるアガロースのゲルビーは、食品機能成分、薬理成分および各種細胞の包括担体として、またクロマトグラフィー用の分離担体として様々な分野で利用されている。内包物質の放出性、内包細胞への栄養や酸素の供給、およびクロマトグラフィーにおける分離性能は、ゲルビーズの粒径の影響を受け、とくに微小かつ均一なゲルビーズの作製が望まれる。しかしながら、平均粒径が50μm より小さく、かつ粒径の均一性の高い寒天ゲルビーズの作製は難しく、効率的な生産につながる手法は確立されていない。

筆者らは、均一性の高い寒天ゲルビーズの作製方法として、マイクロチャネル（MC）乳化法により作製した单分散油中水滴（W/O）エマルションを利用する方法を検討した。すなわち、ゲル化温度以上に保温した寒天水溶液を、乳化剤を含む有機溶媒中にMCを介して圧入することで、液滴径のそろったW/Oエマルションを作製し、これをゲル化温度以下に冷却することで、均一径寒天ゲルビーズを作製することを試みた。MC乳化法は、低剪断場で直径数μm～数百μmの単分散液滴を作製でき、乳化プロセスにおける発熱もほとんどないため、酵素をはじめとする生理活性タンパク質や細胞の内包化にも適していると考えられる。

本研究では、3種類の異なるMC構造を有する平板溝型シリコン製MC基板を使用した。まず、分散相である寒天水溶液への食塩（NaCl）の添加効果について調べた。MC乳化法により寒天含有液滴を均一に作製するためには、十分な浸透圧を付与するための分散相へのNaClの添加が必須であった。0.2 M のNaClを含む1 wt% 寒天水溶液を分散相、5 wt% のSpan 85（ソルビタントリオレアート）を乳化剤として含むイソオクタン溶液を連続相とした場合に、3 種のMC基板を用い平均液滴径15～34μm、変動係数（CV）10%以下の均一径W/Oエマルションを作製することができた。さらに、得られたエマルションを室温まで冷却することで、均一な径をもつ寒天ゲルビーズを作製できることを示した。MCからの液滴形成挙動をハイビジョンカメラにより解析した結果、単一のMCから毎秒数個～二十個程度の液滴が形成されていることがわかった。適切な条件下では、MC基板に分散相が付着することなくスムーズに液滴が形成する様子が観察された。

続いて、乳化挙動に及ぼす寒天濃度および温度の影響を調べた。寒天濃度0.5～2.0 wt%の範囲で、単分散または準単分散な寒天含有液滴を作製することができた。寒天濃度1 wt%の場合、40℃よりも低い乳化温度では均一な液滴の形成が可能であったものの、一部のMCで分散相による閉塞が認められた。このときの寒天水溶液のゲル化温度は約41℃であったことから、安定な乳化を行うためには分散相がゲル化しない温度で操作が望ましいことがわかった。40～50℃の温度帯では変動係数10%未満の均一径液滴を作製することができた。一方、50℃以上では、形成した液滴が直ちに合体することにより大きな液滴となり、液滴径は分散化することが示された。以上の検討により、寒天濃度0.5～2.0 wt%、温度40～50℃の範囲でMC乳化を行うことで、均一性の高い寒天含有W/Oエマルションを作製できることが明らかとなった。