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ORIGINAL ARTICLE

Monoolein cubic phase containing acidic proteinoid: pH-dependent release

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Abstract

Objective: Monoolein (MO) cubic phase containing acidic proteinoid was prepared for a pH-dependent release. *Methods*: The acidic proteinoid was prepared by a thermal-condensation reaction of Asp and Leu (9.85/0.15 in molar ratio). To prepare MO cubic phase, molten MO was hydrated with the proteinoid solution in distilled water. For pH-dependent release experiment, amaranth was included as an anionic dye, and either auramine O or methylene blue was contained as a cationic dye. *Results*: The release of amaranth from the cubic phase was promoted under neutral and alkali conditions, possibly because of electrostatic repulsions between the anionic dye and the ionized carboxylic group of the acidic proteinoid. On the contrary, the releases of auramine O and methylene blue were suppressed under neutral and alkali conditions, probably because of electrostatic interactions between the cationic dyes and the ionized carboxylic group. *Conclusion*: The acidic proteinoid is believed to control the releases in response to change in pH.

Key words: acidic proteinoid, cubic phase, electrostatic interactions, monoolein, pH-dependent release

Introduction

Drug delivery carriers that release their contents in response to environmental stimuli (variations in pH, temperature, chemical concentration, light, fields) have been of great interest to the scientists for two decades, because of their abilities to enhance the bioavailability and the efficacy of a therapeutic agent[1,2]. They are usually called stimuli-sensitive drug carriers. Among them, pH-sensitive carrier is one of the extensively studied carriers for their use in oral vaccination[3], colon-specific delivery[4,5], gene transfection[6], targeting leukemic cells[7], and targeting tumor[8,9]. Liposomes, phospholipid bilayer vesicles, have been frequently employed for the development of pH-sensitive carriers because of their biocompatibility. pH sensitizers such as cholesteryl hemisuccinate (CHEMS) or copolymers of *N*-isopropylacrylamide (NIPAM) and methacrylic acid (MAA) were included in phospholipid bilayers of liposomes[10–13]. The degree of ionization of carboxylic residues has a greater effect on the integrity of the liposomal membrane, leading to a pH-sensitive release. On the other hand, alginate beads coated with chitosan were proposed for a pH-sensitive carrier for oral delivery[14]. Because chitosan has a strong positive charge in acidic conditions, it will be adsorbed electrostatically on the negatively charged surface of alginate bead. The charge intensity of chitosan will be weakened in neutral conditions so the chitosan layers will swell, giving rise to an extensive release[15]. Another example would be proteinoid microspheres. The microspheres prepared by acidic proteinoids were proposed for the use as insulin carrier for oral delivery[16]. The spheres will be stable in acidic gastric fluid, protecting insulin from the harsh gastric conditions. If they were transported into the blood vessel (neutral condition) by penetrating the gastric mucosal membranes, they would be disintegrated by inter-/intramolecular electrostatic repulsions, releasing their contents into blood. Recently, cubic phase was extensively studied because of their versatility as drug carrier. Monoolein (MO) is the most widely used building block for cubic phase. When it is in equilibrium with excess water, bicontinuous-inverted cubic phase is reported to be formed[17–20]. Two intercrossing water channels pass through the cubic phase

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and they are separated by MO bilayers. Hydrophilic compounds could be trapped in the water channels and hydrophobic ones could be loaded in the lipidic matrix of the bilayers. To control the release from the cubic phase, a few approaches have been proposed. Alginate was included in the water channel to obtain calcium ion-sensitive release[21]. Poly(*N*-isopropylacrylamide) was included in the water channel to obtain temperature-sensitive release[22].

In this study, proteinoid prepared using aspartic acid (Asp) and leucine (Leu) (Prot AL) was trapped in the water channels of MO cubic phase to obtain a pH-dependent release. Prot AL is ionizable because of the carboxylic group of Asp residues, and the degree of ionization depends on the pH of the solution. Leu is an amino acid having a hydrophobic dimethylethyl group and it was copolymerized with Asp to obtain a surface-active acidic proteinoid (Prot AL). The surface-active proteinoid is likely to be immobilized onto the surface of the water channel because of the anchoring of the dimethylethyl group into the hydrophobic matrix of MO bilayers. The releases from the cubic phase containing Prot AL were investigated at various pHs using amaranth as an anionic dye, and auramine O and methylene blue as cationic ones.

Materials and methods

Materials

Monoolein (1-monooleoyl glycerol, MO) was gifted by Danisco Ingredients A/S (Copenhagen, Denmark) (monoglyceride content is approximately 95.7% and oleic acid content is approximately 90%). L-Aspartic acid, DL-leucine, amaranth, methylene blue, auramine O, glycerol, sodium citrate, and citric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium bicarbonate was provided by BioShop Canada Inc. (Burlington, VT, USA). *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) was obtained from USB Corporation (Cleveland, OH, USA). Dialysis membrane (MWCO: 1000) was obtained from Spectrum (Rancho Dominguez, CA, USA). All other reagents were of analytical grade.

Preparations and characterizations of Prot AL

Proteinoid composed of Asp and Leu (Prot AL) was prepared following the method described elsewhere[16,23]. About 10 g of Asp was mixed with 3 mL of glycerol, and the mixture was put in 250 mL of a three-neck flask. While purging the flask with N_2 gas, the mixture was melted in a silicone bath thermostated at 160°C. And then 0.15 g of Leu was added to the molten mixture and the reaction was continued at the same temperature for 12 hours. After the reaction mixture was cooled to the room temperature, 40 mL of sodium bicarbonate (10% in distilled water) was put into the flask to dissolve the reaction mixture. The reaction mixture was put into a dialysis membrane (MWCO 1000) and it was dialyzed against 1 L of distilled water with 12 time exchanges for 72 hours. The product was freeze-dried for further use. ^{13}C nuclear magnetic resonance (NMR) spectrum of Prot AL was taken on a Varian VXR-500S spectrometer (Bruker DPX 400MHZ (9.4T), Billerica, MA, USA, located at Central Laboratory of Kangwon National University) using deuterium oxide as a solvent. The Fourier transform infrared (FTIR) spectrum was taken in KBr pellet using Perkin–Elmer Fourier-Transformed Infrared spectrophotometer instrument (EXCALIBER Series, Cambridge, MA, USA). To measure the surface activity of Prot AL, the proteinoid was dissolved in distilled water at various concentrations. The surface tensions of the proteinoid solutions were measured using a ring method with a tension meter (SEO D60A, Surface Electro Optics Co., Ltd., Gunpo, Korea). The pHs of the solutions were adjusted to 3.0, 5.0, 7.0, and 9.0 using 1 N HCl and 1 N NaOH. The three time measurements were made for each sample under a specific condition, and the values were averaged. To observe the effect of Leu on the surface activity of proteinoid, the surface tensions of the solutions of Asp proteinoid without Leu were also measured.

Preparations of cubic phases

About 1 g of MO was put into a vial. The solid mixtures were melted in a water bath, kept at 60°C. About 0.429 g of distilled water containing Prot AL (0.67%) and dye [amaranth (0.1%), methylene blue (0.1%), or auramine O (0.1%)], preheated to the same temperature, was added over the molten MO and then it was kept at 25°C until clear gels were obtained. In parallel, cubic phase free of the acidic proteinoid was prepared as a control for pH-dependent release experiment.

pH-dependent release from cubic phases

The several duplicates of a cubic phase were prepared to determine the amount of dye (amaranth, methylene blue, or auramine O) released at pHs 3.0, 5.0, 7.0, and 9.0 for 100 hours. Five milliliters of buffer solution was laid over the cubic phases contained in 20 mL glass vials and they were whirled at 80 rpm on a shaker (JS Research JSSI-100C, JS Research Inc., Gongju, Korea) at room temperature. Sodium citrate buffer (10 mM) was used for pHs 3.0 and 5.0, and HEPES buffer (10 mM) was used for pHs 7.0 and 9.0. The supernatant was assayed for dye at predetermined time intervals. The amounts of amaranth, auramine O, and methylene blue released from the cubic phase were determined by measuring the absorbance at 520, 432 and 610 nm, respectively, using a UV spectrophotometer (JENWAY 6505 UV/visible spectrophotometer, Bibby Scientific Ltd., Stone, Stafford, UK).

Results and discussion

Characterization of Prot AL

The peaks between 170 and 180 ppm were found in ^{13}C NMR spectrum of Prot AL and they are ascribed to amide

bond. In FTIR spectrum, the peaks in the range of 1000–1250 cm^{-1} are because of the C–N stretching of amino acid residues, a peak around 1690 cm^{-1} comes from the carbonyl group of amide bonds, and a strong peak around 3360 cm^{-1} is attributed to the –NH– of amide bonds. Figure 1 shows the variation in the surface tensions of Prot AL solutions with variations in the concentration and the pH. The surface tension decreased in a saturation manner and the saturations were observed around 0.125%. When the pHs were 7.0 and 9.0, the surface tensions were around 62.5 dyne/cm at the concentration of 0.25%. The decrease in the surface tension was only about 10 dyne/cm and it means that the surface activity of the acidic proteinoid was low at those pHs. Most of the carboxylic groups will be ionized at pHs 7.0 and 9.0 because the p*K* value of carboxylic acid is around 5.3. Thus, the acidic proteinoid will exhibit strong hydrophilicity at those pHs and it would hardly be at air–water interface. This would explain why the surface activities at pHs 7.0 and 9.0 were low. On the other hand, when pH was 5.0, the surface tension was around 52 dyne/cm at the concentration of 0.25%. Because the pH of 5.0 is less than p*K* value, more than 50% of carboxylic groups will be protonated so the hydrophilicity of the acidic proteinoid will be reduced. As a result, the hydrophobicity of the pendant group (trimethylethyl group) of Leu residue would be dominant and it will effectively bring the proteinoid to air–water interface, leading to a marked decrease in the surface tension. The surface tension further decreased when the pH was 3.0. Figure 2 shows the changes in the surface tension of Asp proteinoid solutions with variations in the pH and the concentration. Like the solutions of Prot AL, Asp proteinoid reduced the surface tension at pHs 3.0 and 5.0 more effectively than at pHs 7.0 and 9.0. However, the surface activity of Asp proteinoid was much lower than that of Prot AL. This is possibly because the trimethylethyl group of Leu residue will act as a hydrophobic segment, leading to a higher surface activity.

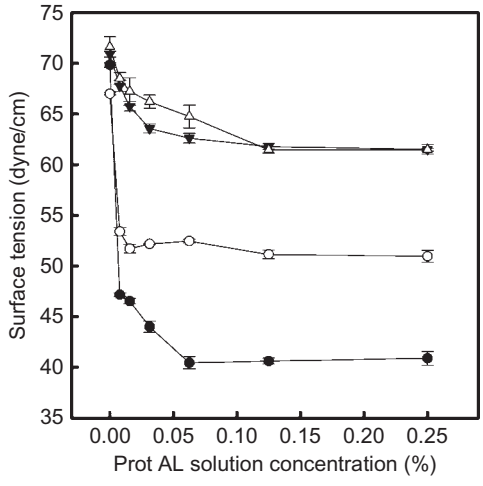

Figure 1. Variation in the surface tensions of Prot AL solutions with varying concentration and pH. pH 3 (●), pH 5 (○), pH 7 (▼), pH 9 (△).

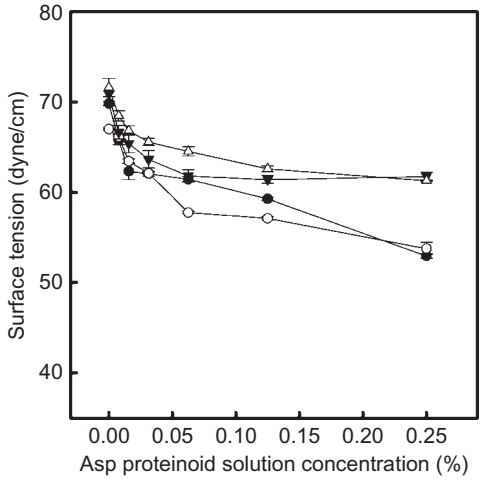

Figure 2. Variation in the surface tensions of Asp proteinoid solutions with varying concentration and pH. pH 3 (●), pH 5 (○), pH 7 (▼), pH 9 (△).

Preparation of cubic phases

Figure 3 shows the photographs of upside-down vials including MO gel, MO gel containing Prot AL, and MO gel containing Prot AL and dye (amaranth, methylene blue, or auramine O). All the preparations were stuck on the top of the vials and they were transparent, indicating that the gels are believed to be cubic phases. Cubic phase is known to be isotropic and optically transparent[24–26].

pH-dependent release of amaranth from cubic phases

The slope of standard curves for amaranth was expressed by $Y = AX$, where Y is absorbance at 520 nm and X is the concentration of amaranth in % (w/w). The slope, A, of the calibration curves for pHs 3.0, 5.0, 7.0, and 9.0 was 376.9, 381.6, 389.6, and 390.6, respectively. Figure 4 shows the release of amaranth from cubic phase containing Prot AL at pHs 3.0, 5.0, 7.0, and 9.0 for 100 hours. In the early stage of the release (for the first 20 minutes), the release increased in a saturation manner and it seemed to be a first-order release. Thereafter, the release increased linearly with respect to time (a zero-order release). The degree of release was higher at a higher pH. For example, % release at pHs 3.0, 5.0, 7.0, and 9.0 in 100 hours was about 52%, 68%, 70%, and 74%, respectively. In neutral and alkali conditions, the acidic proteinoid will be deprotonated so the intensity of negative charge on the chain would be stronger. As a result, the negatively charged amaranth is likely to be expelled by an electrostatic repulsion, leading to a promoted release. Figure 5 shows the release from cubic phase containing no proteinoid at pHs 3.0, 5.0, 7.0, and 9.0 for 100 hours. The release profiles looked similar to those of cubic phase containing proteinoid. However, the release was independent of pH. The degrees of

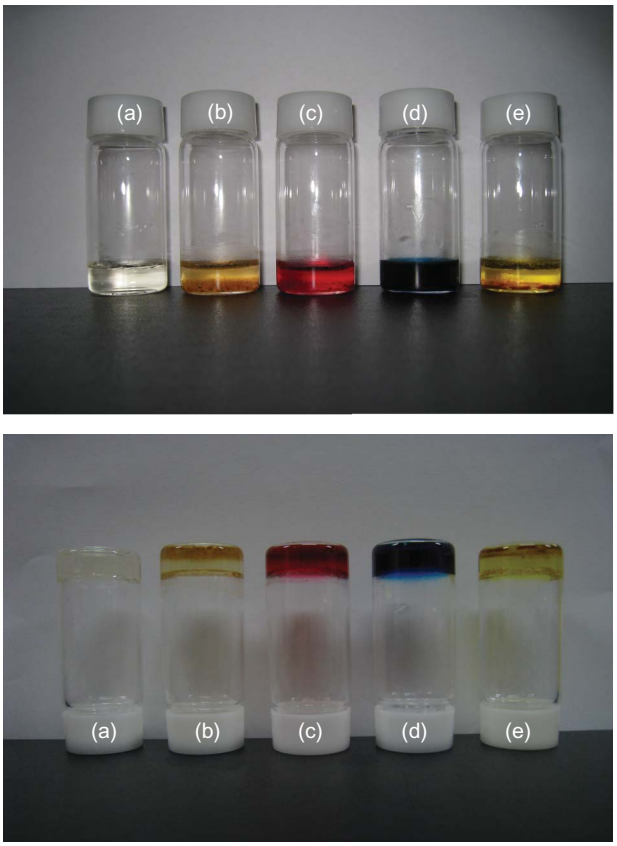

Figure 3. Photographs of upside-down vials including MO gel (a), MO gel containing Prot AL (b), and MO gel containing Prot AL and dye [amaranth (c), methylene blue (d), or auramine O (e)].

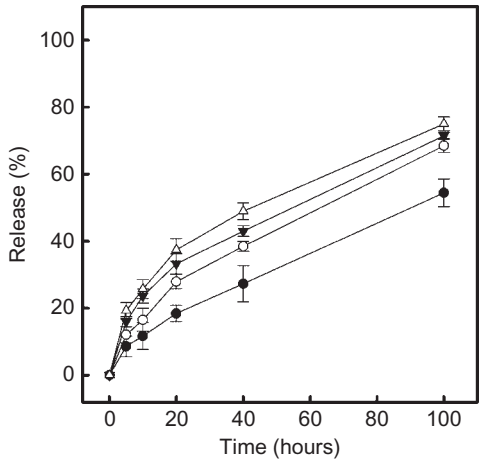

Figure 4. Release of amaranth from cubic phase containing Prot AL at pH 3.0 (●), 5.0 (○), 7.0 (▼), and 9.0 (△) for 100 hours.

release from cubic phase containing no proteinoid were almost the same as that of release from proteinoid-loaded cubic phase in contact with buffer solution (pH 3.0). It means that the proteinoid in cubic phase has little effect on the release of amaranth when the pH of release medium was 3.0. In the acidic condition, the acidic proteinoid will be almost protonated so there

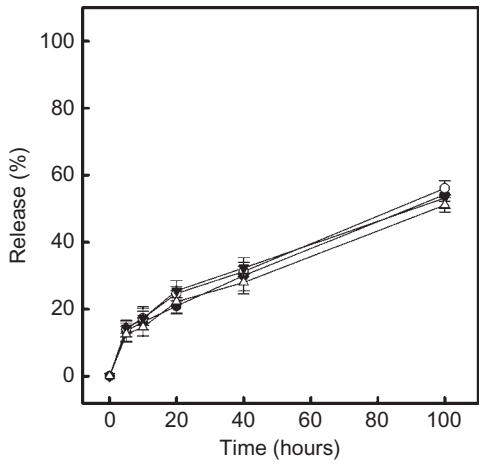

Figure 5. Release of amaranth from cubic phase containing no proteinoid at pH 3.0 (●), 5.0 (○), 7.0 (▼), and 9.0 (△) for 100 hours.

would be little negative charge on the proteinoid chain. Accordingly, there would be no significant electrostatic interaction between the proteinoid and amaranth. This would explain why the proteinoid had little effect on the release at pH 3.0.

pH-dependent release of auramine O from cubic phases

The slope of standard curves for auramine O was expressed by $Y = AX$, where Y is absorbance at 432 nm and X is the concentration of auramine O in % (w/w). The slope, A, of the calibration curves for pHs 3.0, 5.0, 7.0, and 9.0 was 1131.7, 1271.8, 941.4, and 1147.8, respectively. Figure 6 shows the release of auramine O from cubic phase containing Prot AL at pHs 3.0, 5.0, 7.0, and 9.0 for 100 hours. The degrees of release were less than 3% at all the pHs tested, and they were much smaller than those of amaranth even though the molecular weight of auramine O, 303.8, was smaller than that of

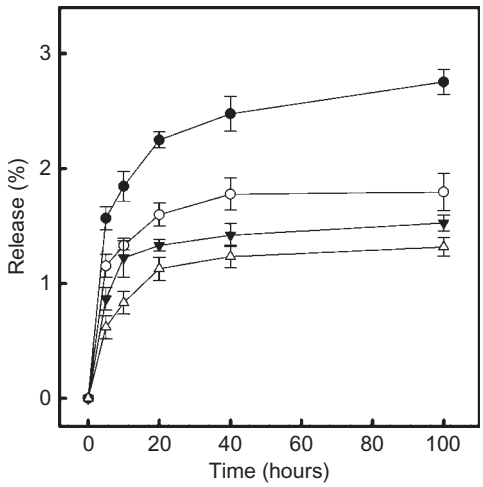

Figure 6. Release of auramine O from cubic phase containing Prot AL at pH 3.0 (●), 5.0 (○), 7.0 (▼), and 9.0 (△) for 100 hours.

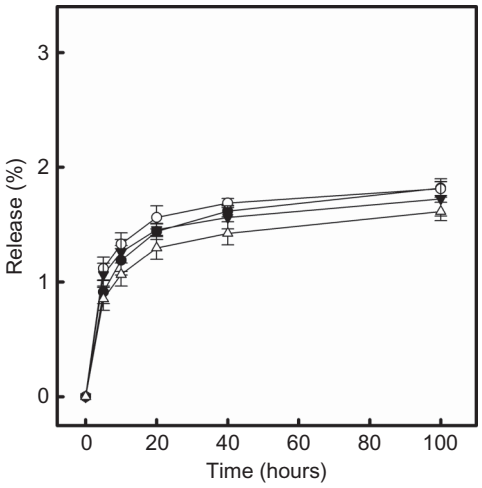

Figure 7. Release of auramine O from cubic phase containing no proteinoid at pH 3.0 (●), 5.0 (○), 7.0 (▼), and 9.0 (△) for 100 hours.

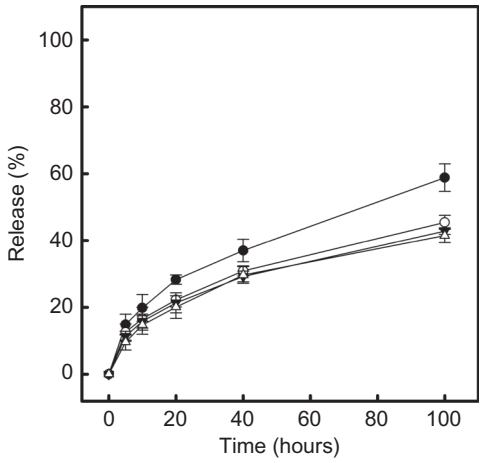

Figure 8. Release of methylene blue from cubic phase containing Prot AL at pH 3.0 (●), 5.0 (○), 7.0 (▼), and 9.0 (△) for 100 hours.

amaranth, 604.5. Auramine O is positively charged dye so it will interact with the acidic proteinoid by an electrostatic interaction. This may account for the lower degree of release. Another reason would be that auramine O has dimethylaniline groups and it would be readily portioned into the lipidic matrix of cubic phases because of the hydrophobic interaction. In this circumstance, the thermodynamic activity of the dye will be low, leading to a suppressed release. In the early stage of the release (for the first 40 minutes), the release increased in a saturation manner, and thereafter there were no significant releases. The degree of release was higher at a lower pH. For example, % release at pHs 3.0, 5.0, 7.0, and 9.0 in 100 hours was about 2.7%, 1.7%, 1.5%, and 1.3%, respectively. This is possibly because the degree of ionization of acidic proteinoid is lower at the lower pH, giving rise to the reduction in the electrostatic interaction between the acidic proteinoid and the positively charged dye. Figure 7 shows the release of auramine O from cubic phase containing no proteinoid. The release patterns were similar to those of cubic phase containing acidic proteinoid. However, the pH of the release medium had little effect on the degree of release. Accordingly, it is believed that the ionizable group (the carboxylic group) of acidic proteinoid controls the release of the dye in response to the change in pH.

pH-dependent release of methylene blue from cubic phases

The slope of standard curves for methylene blue was expressed by $Y = AX$, where Y is absorbance at 610 nm and X the concentration of methylene blue in % (w/w). The slopes, A, of the calibration curves for pHs 3.0, 5.0, 7.0, and 9.0 were 986.5, 901.1, 957.0, and 970.0, respectively. Figure 8 shows the release of methylene blue from cubic phase containing Prot AL at pHs 3.0, 5.0, 7.0, and 9.0 for 100 hours. Like the release of auramine O, the degree of release was higher at a lower pH. For example, the degrees of release at pHs 3.0, 5.0, 7.0, and 9.0 in 100 hours were about 58.5%, 44%, 42%, and 41%, respectively. In acidic conditions (e.g., pH 3.0), the carboxylic groups of acidic proteinoid will be unionized so electrostatic interaction between acidic proteinoid and methylene blue will be low, leading to a high release. On the other hand, even though the molecular weight of methylene blue, 373.9, is higher than that of auramine O, 303.8, the degrees of release of methylene blue were much higher than those of auramine O (also a positively charged dye). Besides electrostatic interaction, hydrophobic interaction could be a major factor to determine the degree of release. The oil–water partition coefficient of methylene blue would be lower than that of auramine O, as the former dye is more polar than the latter one. That is, the thermodynamic activity of methylene blue in the cubic phase will be higher. This may account for why methylene blue exhibited a higher release than auramine O. Figure 9 shows the release of methylene blue from cubic phase containing no proteinoid. The

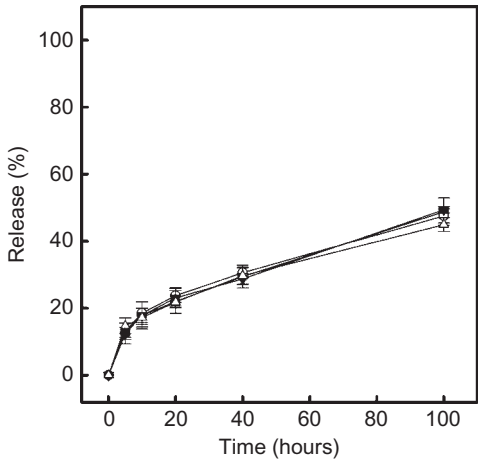

Figure 9. Release of methylene blue from cubic phase containing no proteinoid at pH 3.0 (●), 5.0 (○), 7.0 (▼), and 9.0 (△) for 100 hours.

degrees of release were almost the same, whatever the pHs of release medium were. Accordingly, it is believed that the pH-dependent release of methylene blue from the acidic proteinoid-loaded cubic phase is because of electrostatic interactions between the dye and the proteinoid.

Conclusion

MO cubic phase containing acidic proteinoid (Prot AL) exhibited pH-dependent releases. The release of anionic dye (amaranth) from the cubic phase was promoted under neutral and alkali conditions, whereas the releases of cationic dyes (auramine O and methylene blue) were suppressed under the same conditions. Electrostatic interactions between the charged dye and the acidic proteinoid would be responsible for the pH-dependent release.

Declaration of interest

This work was supported by grant no. 70004549 from Ministry of Knowledge Economy. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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