Polymer(Korea), Vol. 40, No. 2, pp. 258-264 (2016)
http://dx.doi.org/10.7317/pk.2016.40.2.258

ISSN 0379-153X(Print)
ISSN 2234-8077(Online)

저분자 은-알지네이트의 제조 및 항균특성

신철화* · 이봉우** · 최수경***,†

*조선대학교 정책대학원 중독재활복지학과, **한국소방산업기술원 위험물관리부
***조선대학교 공과대학 생명화학고분자공학과

(2015년 10월 15일 접수, 2015년 10월 28일 수정, 2015년 10월 28일 채택)

Preparation and Antibacterial Properties of Low Molecular Weight Silver Alginates

Chul-Wha Shin*, Bong-Woo Lee, and Soo-Kyung Choi***,†**

**Addiction Rehabilitation with Social Welfare, Graduate School of Policy Studies, Chosun University, Gwangju 61452, Korea*
***Hazmat Management Department, Korea Fire Institute of Industry and Technology, Gyeonggi-do 17088, Korea*
****Department of Biochemical & Polymer Engineering, Chosun University, Gwangju 61452, Korea*

(Received October 15, 2015; Revised October 28, 2015; Accepted October 28, 2015)

초록: 본 연구에서는 전자빔 조사에 의한 저분자 알지네이트의 항균 특성을 최대화하기 위해 여러 분자량의 은-알지네이트(SA)의 콜로이드 용액을 제조하였다. 제조된 입자의 크기 분포는 0.1~3.4 μm의 범위로 알지네이트의 분자량이 증가함에 따라 크기가 증가하는 경향을 보였으며 SA의 분자량이 작을수록 대장균, 포도상구균, 여드름균에 대한 항균특성이 우수함을 알 수 있었다. SA로 처리한 모든 시료에서 대조군에 비하여 시험균의 성장속도가 현저히 감소됨을 알 수 있다, 저분자 은-알지네이트는 고분자 알지네이트, 저분자 알지네이트보다 현저하게 높은 항균특성을 보이며 이들의 MIC 값은 대략 350 ppm이었다.

Abstract: To improve the antibacterial efficacy of alginates, the low molecular weight silver alginate (LMWSA) was produced by using depolymerized alginate obtained after electron beam irradiation. The particle size of silver alginates (SA) colloids ranged from 0.1 to 3.4 μm. All the low molecular weight silver alginates (LMWSA) particles showed a reduced growth of the germs tested compared with the control sample. The antibacterial effect of the LMWSA was considerably higher compared with silver-free high molecular weight alginates (HMWA) and low molecular weight alginates (LMWA). The silver-containing LMWSA showed, for example, a minimal inhibitory concentration (MIC) of about 350 ppm value and high antibacterial characteristics in *E. coli, Staphylococuus aureus (S. aureus)* and *Propionebacterium acene (P. acene)*.

Keywords: low molecular weight silver alginate (LMWSA), antibacterial properties, particle size.

Introduction

Alginate is water soluble dietary fiber which has various intrinsic functionalities. Particularly, with excellent heavy metal absorption, it has an outstanding ability in defecation and evacuation of heavy metal from the body. The water soluble alginates have excellent complexation properties for di- and multivalent metal ions and outstanding abilities in defecation and removal of heavy metals from the body. Some alginate derivatives show antibacterial and antioxidant capacities as well as the following unique physical characteristics.

Alginates form a hydrogel on a wounded area and quickly absorb the body fluid. It can be pasted onto a stab wound and when saline water is added, the insoluble calcium salt complexes are converted into the soluble sodium salt, making the gel film easier to peel off. Then, the fiber remaining on the stab wound is finally biodegraded. Silver alginate wound dressings in particular are known to have benefits in wound care, with a recent article highlighting bioavailability of ionic silver for long periods of time.[1]

Therefore, alginate can be used as bioactive substance and medicine for wound healing and wound dressing. Recently, studies have been conducted on antibacterial property of alginate to expand on their applications.[2,3] One of the methods to

†To whom correspondence should be addressed.
E-mail: sookchoi@chosun.ac.kr

improve antibacterial effects is the addition of certain ions, such as Ag^+. Especially, the silver ions are known to generate active oxygen, which has sterilizing effects.[4]

When microorganisms ingest the nanoparticular-silver, particle size 1-100 nm, they can cause respiratory and metabolic dysfunction which can kill various virus, bacteria, fungus, etc. Silver particles within the cytoplasm can induce metabolic dysfunction in bacteria cells suppressing, for example proliferation of the tuberculosis bacillus. In gram positive bacteria, it cell proliferation through the inhibition of cell wall synthesis has been reported.[5] However, it was observed that nanocrystalline (elementary) silver has a negative effect on human HaCaT keratinocytes.[6] Recently, Beele *et al.*[7] created a dressing material for stab wounds by using silver alginate/carboxymethylcellulose and described its properties. A synergetic effect of the two substances in wound dressing and antibacterial activity was observed. As the result, in the property of stab wound dressing, the two substances were shown to work in synergy and to have excellent antibacterial activity. Ionic silver (Ag^+) strongly reacts with -SH (thiol), -COOH, and -OH groups destroying cell membranes of the bacteria or disturb cellular functions through denaturation of proteins.[8,9] It can also influence enzymatic and metabolic processes. Ag-alginate dressing applied to plankton showed significant long-term antimicrobial activity against *Pseudomonas aeruginosa* and even the fungus *Candida albicans*.[1]

The biological activity of polysaccharide is known to be closely related with molecular weight and its distribution.[10] The size and shape of the polysaccharide with high molecular weight causes steric restriction in passing through cell membrane, and this suppresses the expression of the functions. Size and shape of high-molecular mass polysaccarides cause restrained diffusion and permeation through cell membranes suppressing its intracellular activity. This drawback of high molecular mass alginates can be overcome by controlled depolymerized polysaccharides and also chemical modifications are easier. This concept can even be applied to new natural drugs.[11] Besides molar mass and molar mass distribution the degree of substitution block lengths and distribution of the chain-forming repeating units (see Figure 1) control the biological activity of polysaccharides. Consequently, the low molecular weight silver alginates (LMWSA) are a promising candidate for alginate-based, Ag-ions-containing antibiotic agents.

The molecular weight of natural occurring polysaccharides usually maintained high at about 300 kDa or above. High molecular weight alginates (HMWA) is rarely used because of the reasons mentioned before and the high solution viscosity that comes with its high molecular mass. The molar mass is commonly adjusted to approximately 100~10 kDa or much less at 5 kDa. Depending on the type of polysaccharide, beyond 200 kDa it is difficult to dissolve the polymers and the solution viscosity is too high for processing and permeation through membranes.[12-14]

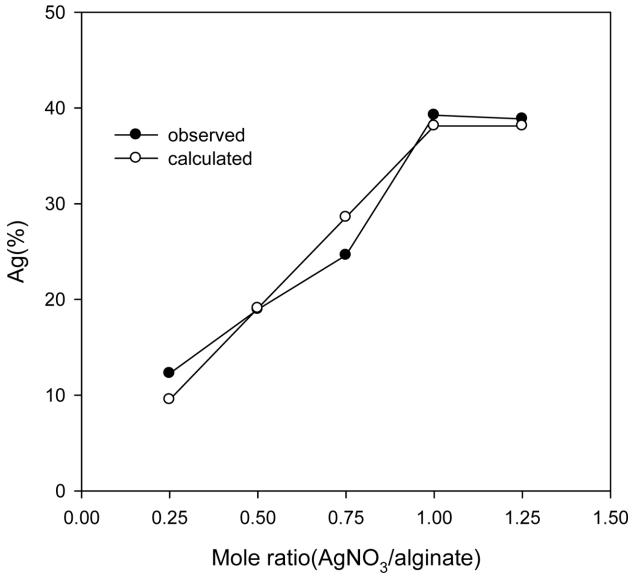

Figure 1. Ag content% *vs*. feed ratio in the LMWSA (M_w=5200).

In previous studies we have reported about various physical and chemical methods of depolymerization of polysaccharides.[15-17,18] Among these, electron beam irradiation proved to be an easy way to control the molecular weight and molecular weight distribution of alginates during depolymerization.[15] In the present study, the LMWSAs were produced by chelating silver with alginate and/or by introducing Ag^+ in the terminal -COOH.

Experimental

Depolymerization of Sodium Alginates. For the depolymerization reaction of SA, e-beam was irradiated on the SA solution (2 w/v%) in hydrogen peroxide atmosphere as shown in Figure 1. Using e-beam irradiation equipment (E-beam process system, EB Tech,Korea), an e-beam of 2.5 MeV in accelerating voltage was applied at dosages of 2.5, 5, 10, 15 and 20 kGy, respectively. The molecular weight of sodium alginates was determined by GPC-MALS (WYATT Technology corporation. Detector-MALLS: DAWN EOS-RI: OPTILAB DSP). As the eluent, 0.1 N $NaNO_3$ aqueous solution was used

at a flow rate of 1.0 mL/min, and the temperature of column oven was 40 °C during the test.[15]

Synthesis and Characterization of Silver Alginate (SA). In the synthesis of SA, the effect of molecular weight of the alginate and the molar ratio of reactant on the silver content and particle size of the product was examined along with their morphology and antibacterial properties. Depolymerized alginate with various molecular weights (M_w; 44800, 30000, 8300, 5200) was used, which was obtained through electron beam irradiation as reported in our previous studies.[15,18] The raw material of high-molecular alginate (HMWA, M_w; 80500) was used as a control material. Feed ratios of silver alginate/alginate were controlled from 0.25 to 1.25. To produce a sample of silver alginate, 3.5 g (20.6 mmol) of silver nitrate (M_w; 169.87, Junsei) was dissolved in 35 mL of deionized water. A dropping funnel was used to drop of sodium alginate (M_R; 198.03) at the speed of 1 mL/min, the 4.0 g (20.6 mmol) of sodium alginate at different molecular weights into the homogeneous solution in 100 mL of deionized water. Then, it was left for 1 h at room temperature for the reaction to occur, and the precipitate was collected. For the effect of dropping rate of the solution on the formation of particles, the dropping rate was adjusted to 2 mL/min. The collected precipitate was washed once with distilled water and three times with ethanol (GR grade), and it was vacuum dried. The yields for the products were more than 60%. For the process and confirmation of reaction, UV-Vis absorption band (Solid-Spec3700, Shimadzu Co.) was examined. The silver content (%) in the product, according to the molecular weight of the depolymerized alginate, was measured by using ICP-AES (PerkinElmer, ELAN DRCII). The particle size and morphology of each sample was measured by using particle size analyzer (PSA, Photal, ELS8000) and SEM (Shimazu, JSM 840-A).

Table 1. Cultivation of Bacteria

Bacteria	Culture condition	Medium
E. coli	37 °C, aerobic	LB medium[a]
Staphylococcus aureus subsp. a	37 °C, aerobic	BHI[b]
Propionibacterium acnes	37 °C, anaerobic	TSB[c]

[a]LB (Luria-Bertani) media. [b]BHI (Brain Heart infusion) media. [c]TSB (Tryotic Soy Broth) media were purchased from BD-Difco™.

Examination of Antimicrobial Effects. For the determination of minimal inhibitory concentration (MIC), as a measure of the antimicrobial efficiency, sample liquid (pH 5.9~6.0) was added to LB (BD-Difco™) broth so that different final concentrations were obtained. Then, the consistent amount of bacteria (i.e. 104-105 CFU/mL) was inoculated and was cultured for 1-5 days at an optimal condition for bacterial growth. The growth curve of the bacteria was obtained based on the optical density (OD, λ=600 nm) of the culture medium. The MIC was read as the least concentration of the antimicrobial agent that was sufficient to completely inhibit visible bacterial growth. When the optical density measurement was not achievable, the medium was diluted in physiological saline (0.85% NaCl) and was spread evenly on agar plate medium, which was cultured for 24 h at 37 °C. The number of colony

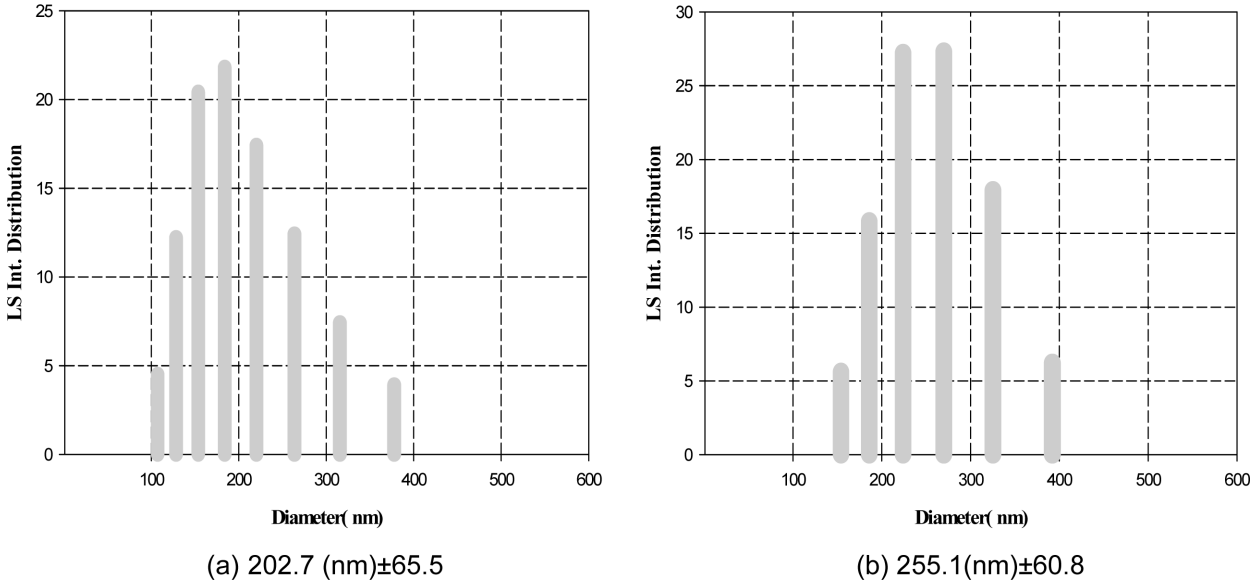

Figure 2. Particle size distribution of (a) LMWSA (M_w=5200); (b) silver alginate (SA-5-0.50).

forming units (CFU) on the agar was counted, and the minimum concentration that has the constant number of bacteria at the inoculation of test bacteria. After culturing, the number of colonies was set as MIC value. HMWA, LMWA and SA with different molecular weights were tested for antibacterial activity in *E. coli*, *S aureus subsp. a, and P acnes.* The culturing conditions and methods for each strain are shown in Table 1.

Aerobic Culture: For the aerobic culture, appropriately diluted (stepwise dilution of original solution, 1~2 mg/mL) samples (HMWA, LMWA and SA) were collected and dissolved in broth. After treating with UV for sterilization for 1 h at clean bench, it was diluted from 10000 ppm. The 50 μL of active bacteria such as *E. coli K12*, and *S. aureus subsp.*[a] (ATTC®25923) were inoculated in each and MIC was measured. Also, cell concentration over time at each sample's MIC concentration was measured. The seed culture, which was cultured for 24 h, was applied to solid culture (culture made by adding 20 g/L of Agar to the broth) at 20 μL each. After 24 h of culturing, colonies were examined.

Anaerobic Culture: The anaerobic culture was processed using the same method as aerobic culture, except that the bacteria, *P. acnes*(ATTC®6919), was inoculated inside of the anaerobic chamber while infusing gas mixture (80% N_2, 10% CO_2, 10% H_2) and was cultured by using gas pack anaerobic system (Difco Co.).

Results and Discussion

Silver Content and Particle Size of SA. A series of SAs was produced by reacting $AgNO_3$ solutions with sodium alginate solution of various molar masses produced by electron beam irradiation as described before.[11,16] The silver content was controlled by the feed ratio. All SAs formed colloidal solution with the particle size controlled by the feed rate (dropping rate of the$AgNO_3$ to the alginate solution). The results are summarized in Table 2. The absorption peak for $AgNO_3$ solution disappeared at UV absorbance of 301 nm, and a new peak appeared at 407 nm indicating surface plasmons caused by the chelation between -OH or -COOH of alginate and Ag^+.

The molar feed ratio $AgNO_3$/alginate was varied in 0.25 steps from 0.25 to 1.25. The feed rate of $AgNO_3$ was usually kept constant at 1 mL/min, see also below. Silver content, size of the colloidal particles and antibacterial properties were determined. The Ag-content of the alginates increases linear with the Ag^+ feed leveling off beyond a molar ratio of 1:1, which means a maximal silver content of 40%, see Figure 1. The feed rate was doubled for sample SA-6-1.00 with the result of a drastically increased particle size (0.485 μm) because of a high chelating rate, which leads to rapid aggregation. Silver contents in the SAs, SA-5-0.25 (molar ratio, 0.25) and SA-5-1.00 (molar ratio, 1.00) were 12.27 and 39.26%, respectively.

As shown in Table 2, the experimental Ag^+-content in the samples SA-1-0.75, SA-1-1.00, and SA-1-1.25 was larger than the theoretical value basing on a simple salt-formation with the alginate -COO^-. This is because the unique metal adsorption property of alginate causes displacement reaction of silver ions as well as silver particle chelation with alginate moiety; there-

Table 2. Ag Contents and Mean Particle Size of Silver Alginates (SAs) in Various MW and Feed Ratios*

Silver alginate (SA)	M_w	Feed mole ratio $AgNO_3$/Alginate	Ag% Observed[a]	Ag% Calculated[b]	Mean particle size (μm)*
SA-1-0.75	80500	0.75	30.07	28.59	1.429
SA-1-1.00	80500	1.00	39.05	38.12	1.482
SA-1-1.25	80500	1.25	40.13	38.12	1.876
SA-2-0.75	44800	0.75	25.62	28.59	0.511
SA-3-0.75	30000	0.75	29.73	28.59	0.338
SA-4-0.75	8300	0.75	25.72	28.59	0.739
SA-5-0.75	5200	0.75	24.62	28.59	0.523
SA-5-0.50	5200	0.50	18.96	19.07	0.255
SA-5-0.25	5200	0.25	12.27	9.53	-
SA-5-1.00	5200	1.00	39.26	38.12	0.380
SA-6-1.00	5200	1.00	38.86	38.12	0.485

*Dropping ratio of $AgNO_3$ was 1 mL/min except SA-6-1.00. SA-6-1.00; dropping rate of $AgNO_3$ solution was 2 mL/min.
[a]Obtained from ICP analysis. [b]Ag content (%) in SA calculated with 100% Ag replacement.

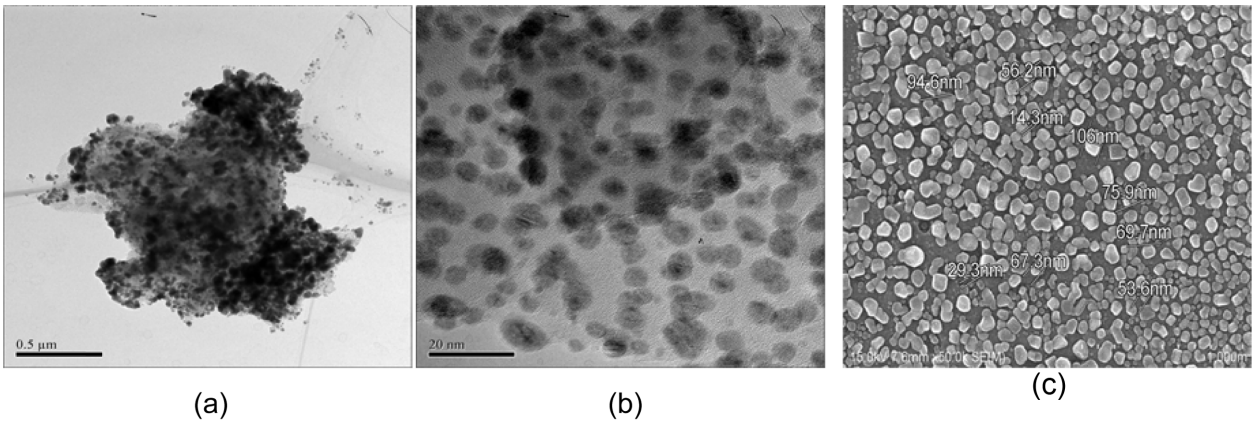

Figure 3. SEM images of (a), (b) SA-1-1.00; (c) SA-5-0.50 colloidal particles.

fore, the experimental value of silver content was obtained to be greater than the calculated value. In the low molecular weight alginate (M_w; 5200), the amount of $AgNO_3$ did not have a significant effect on silver content in SA (Figure 1), and the silver content was shown to be lower than the calculated value. In the case of high molecular weight alginate (SA-1-1.25) treated with the $AgNO_3$ /alginate molar ratio of 1.25, the silver content (40.13%) was shown to be greater than the calculated value (38.12%). This can be considered to be due to an excessive amount of silver nitrate that has chelated with alginate, which is present in the aggregated long molecular chain; and unlike the low molecular weight alginate (sodium alginate), it could be stably present within the chain.

The particle size distribution was within the range of 0.1~3.4 μm, and the size was increased with increasing molecular weight of the alginate. The particle size of the alginate used to create the SA is 202.47 nm, the particle size of the SA-5-0.50 (molar ratio, 0.5) is grown in 255.1 nm(Figure 2). The values shown in Table 2 give the average size of particles that were measured by using PSA. It can be predicted that the particle size was increased due to the silver particle entrapped in the entangled long chain and the molecular chain aggregation. This can be seen in SEM image in Figure 3 which can explain in the same effect. In the case where excessive amount of $AgNO_3$ is treated with LMWA, there was no significant effect on the increase in particle size of SA. However, (Figure 3(c)) when excessive amount of silver nitrate was treated with HMWA (M_w; 80500), the particle size was increased due to the aggregation of long molecular chain of SA (Figure 3(a, b)). The silver content in the repeating unit of the products did not show significant change with the molecular weight of alginate depolymerized, and it was close to the calculated value. This is because the sodium alginate that was not replaced with silver was dissolved in water, and the complex was formed by chelating with SA replaced by silver. This is considered to have caused the silver content within the repeating unit to show relatively greater than the calculated.

Antibacterial Property. To compare the antibacterial property of SA, antibacterial experiments were conducted on LMWA (M_w; 5200) and HMWA (M_w; 80500). MIC of each was measured on *E. coli, S. aureus,* and *P. acene*. The results are shown in Table 3 and Figure 4. The MIC of SA was 350 ppm (Figure 4(a)), while MIC of LMWA was 2000 ppm; and the HMWA showed a MIC value of 5000 ppm (Figure 4(b)). The SA-5-1.00 which was obtained at 1:1 molar feed ratio and contained 39.26% silver showed a MIC value, 350 ppm in common in *E. coli, S. aureus,* and *P. acene* which is a pretty low value. The antibacterial persistence of LMWSA

Table 3. Anti-Microbial Properties of Silver Alginate (SA)

Bacteria	MIC(ppm)		
	SA-5-1.00 (M_w; 5200)	Sodium alginate (M_w; 5200)	Sodium alginate (M_w; 80500)
E. coli.	350	2000	5000
Staphylococcus aureus subsp. a	350	2000	5000
Propionibacterium acnes	350	2000	5000

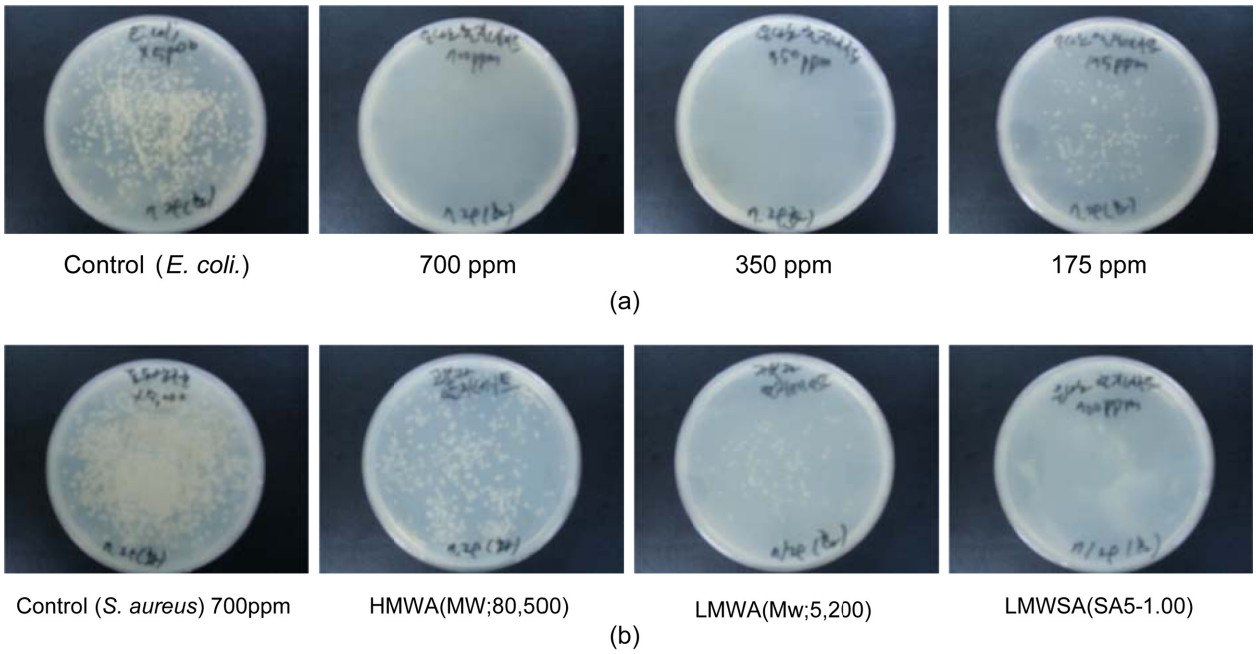

Figure 4. MIC of (a) LMWSA (SA5-1.00); (b) comparison of antibacterial properties of HMWA, LMWA, LMWSA at 700 ppm.

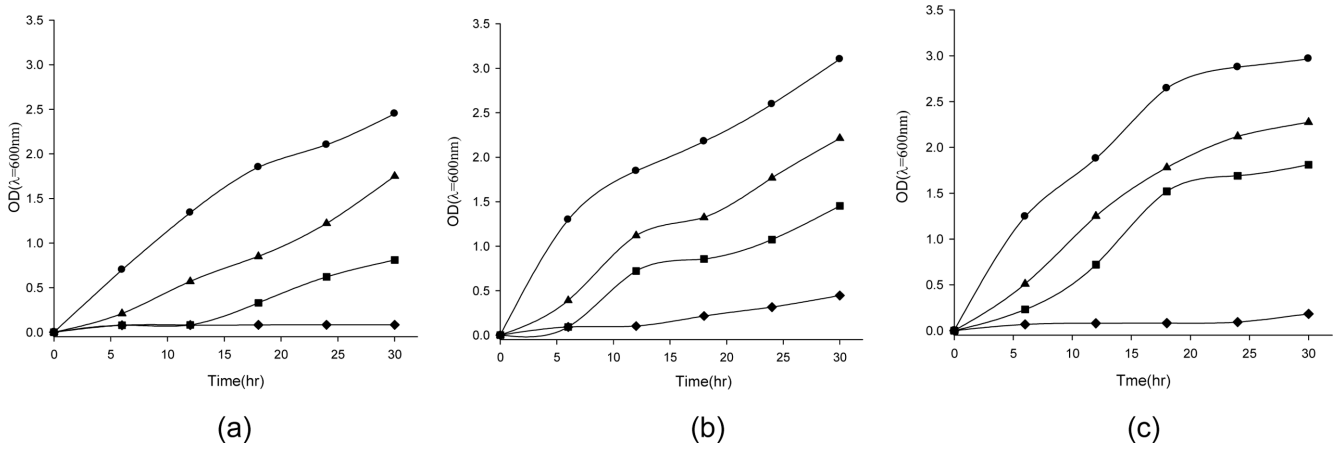

Figure 5. Antibacterial sustainability of alginate and silver alginate against (a) *E. Coli*; (b) *S. aureus*; (c) *P. acnes* at MIC. (● control, ▲ HMWA (M_w=80500) at 5000 ppm, ■ LMWA (M_w=5200) at 2000 ppm, ◆ LMWSA at 350 ppm).

was compared with the HMWA and LMWA. In the MIC concentration of each sample, the time dependence of optical density (OD) value (λ=600 nm) was plotted in Figure 5. In the case of LMWA with its relatively low molecular weight (M_w; 5200) and high acidity (pH 5.4), the antibacterial effect was good for up to 12 h. In the case of LMWSA, the antibacterial effect was prolonged to 30 h. Sridhar *et al.*[19] and Lee *et al.*[20] have reported about antibacterial effect in marine algae and natural alginate in general. The groundbreaking antibacterial property of LMWSA gives access to a tremendous potential in developing medicine for stab- and related wounds, which promise much enhanced antibacterial efficacy.

Conclusions

Using depolymerized alginate obtained from electron beam irradiation, low molecular weight silver alginate was produced. The correlation between their molecular weight, reactant molar ratio, silver content, and particle size was examined. Furthermore, their antibacterial effect on *E. coli, S. aureus,* and *P. acene* was examined, and the following conclusions were made.

1) The particle size of SA was decreased with decreasing molecular weight of the utilized alginate.

2) The particle size distribution of the SA produced was in the range of 0.1~3.4 μm and can be controlled by the feed rate.

3) MIC values for the tested bacteria of LMWSA were all around 350 ppm, which was shown to have good antibacterial property compared to the high molecular weight alginate.

4) Growth delay of bacteria by low molecular weight was 30 h, which was much higher than that of high molecular weight alginate.

Acknowledgement: Thanks for the financial support by NRF under grant 2014R1A1A2058323.

References

1. C. Bradford, R. Freeman, and S. L. Percival, *J. Am. Col. Cerif. Wound Spec.*, **1**, 117 (2009).
2. C. Ma, L. Liu, W. Hua, Y. Cai, and J. Yao, *Fib. Polym*., **16**, 1255 (2015).
3. K. Norajit and G.-H. Ryu, *J. Food Process. Preserv.*, **35**, 387 (2011).
4. R. D. Wolcott, D. D. Rhoads, M. E. Bennett, B. M. Wolcott, L. Gogokhia, J. W. Costerton, and S. E. Dowd, *J. Wound Care*, **19**, 52 (2010).
5. W.-K. Jung, H.-C. Koo, K.-W. Kim, S. Shin, S.-H. Kim, and Y.-H. Park, *Appl. Environ. Microbiol.*, **74**, 2171 (2008).
6. C. Wiegand, T. Heinze, and U. C. Hipler, *Wound Repair Regen*., **17**, 511 (2009).
7. H. Beele, F. Meuleneire, M. Nahuys, and S. L. Percival, *Int. Wound J.*, **7**, 262 (2010).
8. W. K. Jung, H. C. Koo, K. W. Kim, S. Shin, S. H. Kim, and Y. H. Park, *Appl. Environ. Microbiol.*, **74**, 2171 (2008).
9. Q. L. Feng, J. Wu, G. Q. Chen, F. Z. Cui, T. N. Kim, and J. O. Kim, *J. Biomed. Mater. Res.*, **52**, 662 (2000).
10. Q. Wu, C. Zheng, Z.-X. Ning, and B. Yang, *Int. J. Mol. Sci.*, **8**, 670 (2007).
11. G. Franz and S. Alban, *Int. J. Biol. Macromol.*, **17**, 311 (1995).
12. Y. J. Kim, Y. J. Yoo, and H. Y. Lee, *Biotechnol. Lett.*, **17**, 345 (1995).
13. P. Gasesa, *Carbohydr. Polym.*, **8**, 161 (1988).
14. Y. Qin, *Polym. Adv. Technol.*, **19**, 6 (2008).
15. C.-W. Shin and S.-K. Choi, *Appl. Chem. Eng.*, **25**, 227 (2014).
16. Y.-S. Choi and S.-K. Choi, *Polym. Korea*, **35**, 444 (2011).
17. B.-W. Jo and S.-K. Choi, *Carbohydr. Polym.*, **111**, 822 (2014).
18. B.-W. Jo and S.-K. Choi, Korea Patent 10-1148383 (2012).
19. K. R. Sridhar and N. Vidyavathi, *Acta Hydrochim. Hydrobiol.*, **19**, 455 (1995).
20. H. S. Lee and J. H. Suh, *KSBB J.*, **17**, 63 (2002).