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Minicell Formation of *Leuconostoc Mesenteroides* in Pharmaceutical Science

Tuan Q. Nguyen, & Tu K. H. Nguyen*
School of Biotechnology, Hochiminh City International University – Vietnam National University in Hochiminh city.
Quarter 6, Linh Trung ward, Thu Duc district, Hochiminh city.
*Corresponding Author

Abstract

Leuconostoc mesenteoides is commonly used in fermented food. The study on developing molecularly targeted drugs to achieve a higher grade of drug delivery system that is one of the indispensable issues in pharmaceutical fields. This paper reported the effects of carbon sources including in glucose, maltose, lactose, saccharose at the different concentrations as 0, 5, 10, 20, 30 g/L on the cell differentiation of *Leuconostoc mesenteroides* VTCC-B-871. As results, *L. mesenteoides* VTCC-B-871 formed minicells with highly significant number of 4.6 ± 0.3 (%) starting cells in modified MRS broth with 20 % glucose. The minicells were collected and checked for the less than 400 nm in size and round shape under scanning electron microscope. Minicells ($6x10^5$) showed effect against *Pseudomonas aeruginosa*, equaled to 0.85 µg of silver nitrate ($AgNO_3$) in agar diffusion test. Moreover, minicells ($6x10^5$) could package with 0.069 µg $AgNO_3$, approximately. Therefore, minicell could be used as a nanoparticle as well as a potential drug delivery in pharmaceutical science.

Keywords: *Leuconostoc mesenteroides*, minicells, scanning electron microscopy, antimicrobial activities, silver nitrate, drug delivery

1. Introduction

Nowadays, healthcare is one of the most considerable problems all over the world. Due to the availabilities of several medicinal treatment modalities, mortality rates have been reduced significantly. However, it is undeniable that there were several existences such as drug resistance, dose-limiting toxicity, toxic side effects and difficulties of targeted delivery, which cause damages to normal cells as kidney and liver cells. These problems are daunting challenges in terms of medical treatment. Therefore, the combination of pharmaceutical science with advances in cell biotechnology, chemical science and bioinformatics is required to limit the obstacles on the drug development.

The development of many novel drug delivery strategies has been stimulated by the rapid advent of nanotechnology in recent years (Wang et al., 2007). The therapeutic agents are dissolved, entrapped, encapsulated or attached to a nanoparticle which is applied as nanoscale drug delivery vehicles have shown the advantage for directing the drugs to specific target by attaching specific ligands on to their surface, improved stability and therapeutics index and reduce side effects, but increasing the circulation time and bioavailability by manipulating the particle size and surface characteristics of nanoparticles.

Nanoparticles applied as drug delivery systems are submicron sized particles (10 to 1000 nm) (Shim and Turos, 2007) that can be made using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), magnetic, even inorganic or metallic compounds (silica, iron) and bacteria (bacterially derived nanoparticles or "minicells") (MacDiarmid and Brahmbhatt, 2011; MacDiarmid et al., 2007b, 2009).

However, there were several important limitations that have been highlighted and identified in the development of drug delivery system. The ineffective distribution in cells and tissues, limited oral availability, and retention in by passing organs and by macrophages of the reticuloendothelial system after systemic administration (Yun et al., 2012) are typical examples. Beside the enhanced efficacy demonstrated by many targeted nanoparticles, they also face main restrictions: a major dose loss due to receptor- mediated endocytosis and subsequent lysosomal digestion, immunogenicity and non-specificity of the target ligand resulting in accelerated blood clearance, and further impaired cell penetration such as tumor cells compared to the non-targeted nanoparticles (Chen et al., 2012).

The minicell producing strains have been isolated from *Escherichia coli* (Alder et al., 1967), *Bacillus subtilis* (Reeve et al., 1973), *Salmonella typhimurium* (Sheehy et al., 1973), *Haemophilus influenza* (Sedgwick et al., 1975) and the other Gram-positive bacteria (*Listeria monocytogenes*) and Gram-negative bacteria (*Shigella flexneri* and *Pseudomonas aeruginosa*). However, exploiting Lactic acid bacteria (LAB) in drug delivery weren't much studied. LAB help to improve nutritional value of food, control of intestinal infections, improve digestion of lactose, control of some types of cancer and control of serum cholesterol levels. Scientists developed natural antimicrobial products for bio-control of pathogens and have exploited LAB for the competitive exclusion of pathogens and delivery of vaccines and bioactive compounds (Grasson, 2002). Furthermore, LAB play an important role in the treatment of people suffering with tumors and immune compromised subjects. The evidence that LAB effects on human health is remarkable and fascinating for effective utilization. They seem to have relatively low toxicity compared to other treatments (Wood, 1992). The LAB are frequently used as probiotics to improve some biological functions in the host. They also affect on immune system by sending signals to active immune cells through different mechanisms (Perdigon et al., 2001). Altogether, LAB have health promoting attributes, including anti-carcinogenic, anti-allergic effects, hypocholesterolemic properties, immunomodulatory effects and gastrointestinal health improving (Masood et al., 2011; Ongol 2012).

Therefore, the main aim of this study was to develop a new nanoparticle and nano-sized carrier derived from *Leuconostoc mesenteroides* VTCC-B-871, being a lactic acid bacterium showing gram positive cocci. Additionally, the preliminary study on the potential abilities of *Leuconostoc mesenteroides* VTCC-B-871 to be packaged by silver metal

ions was researched. With the aim of generation the silver nanoparticle due to many medical advantages of silver which had been known for over 2000 years. Moreover, the silver compounds have been utilized in numerous antimicrobial applications since the nineteenth century.

2. Materials and Methods

2.1. Bacterial Strains And Media

Leuconostoc mesenteroides VTCC-B-871 obtained from Vietnam Type Collection Culture (Academic Institute, Hanoi, Vietnam). *Leuconostoc mesenteroides* was grown in Lactobacilli MRS broth (De Man et al., 1960).

Pseudomonas aeruginosa (*P. aeruginosa*) ATCC 27853 was used as pathogen indicator in antimicrobial activity test. *Pseudomonas aeruginosa* was grown in LB.

2.2. Design Conditions For Minicell Production By *Leuconostoc*

With the aim of studying on the impact of various carbon sources on the minicell formation, this study implemented the experiments on different kinds of sugar with different concentration in the bacterial culture medium. The bacteria were inoculated into the modified Lactobacilli MRS broth which containing each kind of sugar separately (glucose, sucrose, maltose), in altered concentration (0%, 5%, 10%, 20%, 30%) for 48hrs in order to produce minicells.

Subsequently, the modified Lactobacilli MRS media that provide the high yield of minicells would be applied continuously to study the influence of the temperature on minicell production. *Leuconostoc mesenteroides* were cultured into the selected Lactobacilli MRS broth and incubated at room temperature because of the time limitation and the incubation condition. Moreover, the room temperature is the best condition for *Leuconostoc mesenteroides* survival.

2.3. Minicell Isolation

The cultured bacteria for minicell production were subjected to the minicell isolation for removal of contaminating the parent bacterial cells and cellular debris. Firstly, to separate partially the large parent cells, the culture was centrifuged at 3500 rpm for 20 minutes. Then, the supernatant was collected to undergo further the first and second filtration through the 0.45 µm filter membrane to separate completely minicells. Finally, the filtered supernatant was centrifuged at 14000 rpm for 20 minutes in order to collect the minicells.

2.4. Microscopic Studies For Morphological Characterization Of Minicell

2.4.1. *Light microscopy*

The isolated minicells were observed by microscopic for the morphological alterations and cell counting under a light microscopy with a total magnification of 100X using a Neubauer hemocytometer. The minicell amount was obtained by counting in five small squares (the four 1/25 sq. mm corners plus the middle square) in the central area into focus at low power. The number of counted minicells per unit volume of a suspension was calculated as following equation.

$$\text{The density of obtained minicells (particles/mL)} = \frac{\text{The number of counted minicells}}{\text{The combined volume of five squares (mL)}}$$

2.4.2. *Scanning electron microscopy*

The isolated minicells were examined at Scanning Electron Microscopy Laboratory Room, Vietnam Academy of Science and Technology, 01 Mac Dinh Chi Street, District 1, Ho Chi Minh City to observe the morphology and size of minicells by using the Scanning Electron Microscope (SEM, S-4800, Hitachi Japan) at 10 kV.

2.5. Minicell Packaging Silver Nitrate ($AgNO_3$)

In order to analyze minicell ability to be packaged with metal ions. In this case, the silver metal ions were used to produce silver nanoparticles which have been used as anti-inflammatory and antimicrobial agent (Melaiye et al., 2005 and Rai et al., 2009) and have many applications in DNA detection (Liu et al., 2005), cancer therapy (Singh et al., 2008), optics, catalysis and micro-electronics. The solution (1mL) containing collected minicells was added into 50 mL aqueous solution of 1mM $AgNO_3$ and incubated the suspension at room temperature for 20 hrs to produce completely silver *Leuconostoc* minicells. The incubated mixture had been centrifuged for 20 minutes at 14000 rpm before were washed with phosphate buffer saline (PBS) solution for three times. The silver packaged minicells were collected and then re-suspended in phosphate buffer saline (PBS) to prepare for microbial inhibition assay.

2.6. Microbial Inhibition Assay

The agar diffusion method was applied in order to test the antimicrobial effects of minicells ($6x10^5$), silver-packaging minicells ($6x10^5$), and 20 µL of 1mM $AgNO_3$ or 0.34 µg on *Pseudomonas aeruginosa (P. aeruginosa)* ATCC 27853. These samples were dropped on the sterilized paper discs (5 mm in diameter) placed on the petri plates. The plates were incubated at suitable temperature for 18- 24 hrs. Subsequently, the inhibition zone diameter was measured. Phosphate buffer saline (PBS) solution was used as references. The potency of minicell in antimicrobial activity equaling to $AgNO_3$ was calculated following the formulation:

$$\text{The potency of minicell in antimicrobial activity (µg)} = \frac{\text{Diameter of minicells}}{\text{Diameter of AgNO3}} \times \text{amount of AgNO3}$$

The amount of $AgNO_3$ packaged in minicells were calculated following the formulation:

$$\text{The amount of AgNO}_3\text{ packaged in minicells (µg)} = \frac{\text{Diameter of minicells packaged with AgNO3} - \text{Diameter of minicells}}{\text{Diameter of AgNO3}} \times \text{amount of AgNO3}$$

2.7. Data Analysis

The results of triple replicates for all experiments were expressed as mean± standard deviation and then analyzed using one-way ANOVA and post-hoc Tukey's test for paired comparisons of means. The statistically significant differences were considered with $p < 0.05$.

3. Results And Discussion

3.1. Morphological Differentiation

Leuconostoc has a normal size less than 1 µm (Figure 1) that can be used as nanoparticle. However, this bacterium is a living organism that can replicate and survive in human and animal. Therefore, to apply this bacterium as an ideal nanoparticle, the cell division inhibition is necessary. The study tried to find out the effect of sugar on the cell division. *Leuconostoc* was let to grow in different carbon sources (glucose, sucrose and maltose) with the optimal concentrations (0%, 5%, 10%, 20%, and 30%). The morphological differentiation of *Leuconostoc* was check under light microscope (Figure 2).

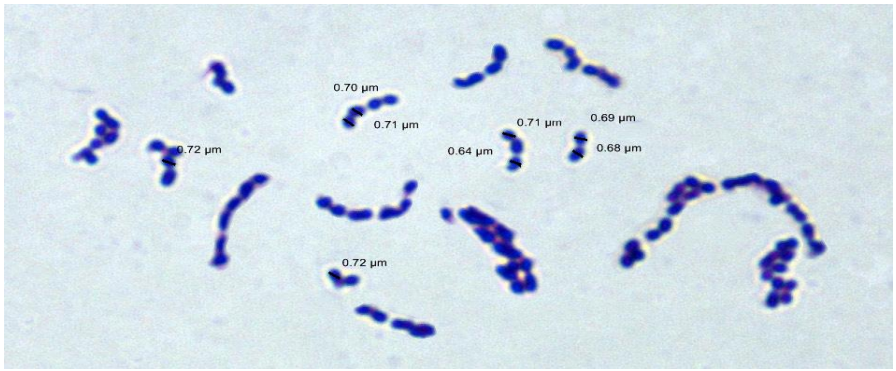

Figure 1. The morphology of *Leuconostoc* under light microscope with the size determination.

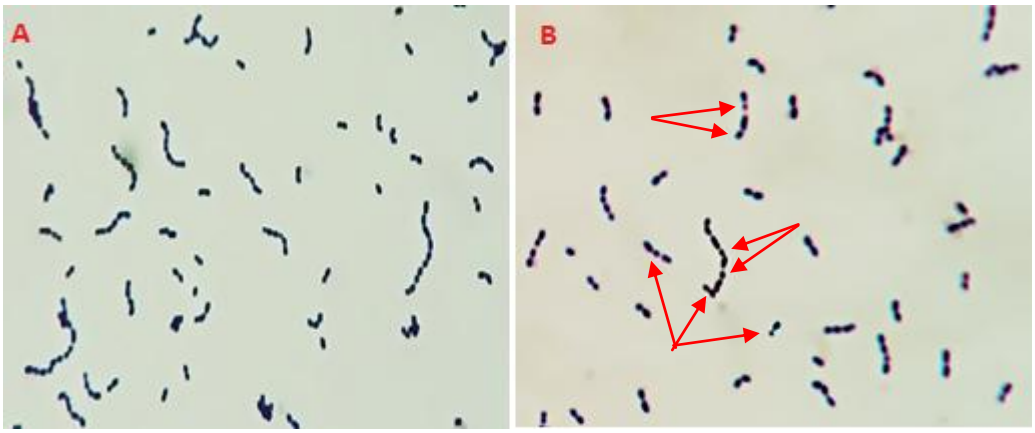

Figure 2. Photomirograph of *Leuconostoc* and its minicells (100X): (A) The morphology of *Leuconostoc* in basic MRS culture medium; (B) The formation of minicells (red arrow) in the modified MRS medium with glucose 20% w/v.

The differentiation occurred in the modified media with sugars because the sugar affected on the cell division inhibition proteins such as FtsZ, a GTPase which well interacted with glucose; or MinD, an ATPase improved the cell division inhibition process when supplied with sugars (Nguyen et al., 2013). Also, to understand well the reasons of differentiation, more studies should be done in future.

3.2. Minicell Production Evaluation By *Leuconostoc* With The Sugars As Carbon Resources

The abnormal small cells of the bacterium were called minicells (Alder et al., 1966). The minicells produced in different sugar containing media were collected, concentrated by filtration, centrifugation. The minicells were checked the size using SEM (Figure 3). The SEM images of minicells with their diameter ranged 400 nm. As a consequence, minicells were generated successfully as nano-size cells. The nanoparticles were utilized with their size up to 1000 nm (Shim and Turos, 2007) for drug delivery in the practice. Therefore, these minicells were considered continuously to develop for drug delivery. Besides, the isolated minicells were demonstrated the absence of bacterial colonies by inoculating on Lactobacilli MRS agar discs and incubating at 37°C for 18- 24hrs. After checking the size of minicells, minicells collected from different sugar conditions were quantitated (Table 1) and analyzed (Figure 1).

Table 1. The proportions of produced minicells by *Leuconostoc* in modified MRS medium containing each kind of carbon sources (selected sugars) in different final concentrations.

The percentage of minicell production (%)					
Sugars	**The sugar concentrations in the culture medium**				
	0%	**5%**	**10%**	**20%**	**30%**
Glucose	0.7 ± 0.2	2.2 ± 0.2	4.1 ± 0.3	4.6 ± 0.3	3.4 ± 0.3
Sucrose	0.7 ± 0.1	1.5 ± 0.3	2.6 ± 0.2	3.4 ± 0.2	1.9 ± 0.3
Maltose	0.7 ± 0.1	1.4 ± 0.2	2.3 ± 0.2	2.9 ± 0.1	1.5 ± 0.1

The analyzed data was means ± SD

The minicells were collected, concentrated by filtration, centrifugation and then calculated. The analyzed data which was shown in Table 1 identified that carbon sources affected significantly on the minicell generation and the minicells were produced as a consequence of the sugar concentration ($p<0.05$). Table 1 showed clearly that minicell production changed very low, closed to zero (0.7%) when *Leuconostoc* was cultured in medium without sugar. At all levels of sugar concentrations (from 5% to 30%) that were presented data, the maximum number of minicells was obtained when using glucose as a carbon source. It was followed by the amount of minicells which were produced in glucose MRS medium. The lowest percentage levels of minicell generation were similar in sucrose and maltose MRS medium by 1.9 and 1.5 percent.

As a result from Table 1, the amount of obtained minicells was increased considerably when *Leuconostoc* was cultured in the modified Lactobacilli MRS media for all tested sugars with final sugar concentrations from 5% to 20%. The obtained minicells were the highest amount for each of kind of sugar at level of factor treatment of 20% sugar concentration. In the modified Lactobacilli MRS medium including glucose, sucrose and maltose, the percentage of generated minicells was 4.6 ± 0.3%, 3.4 ± 0.2% and 2.9 ± 0.1%, respectively. The quantity of produced minicells decreased at very high sugar concentration (30%) by falling to 3.4 ± 0.3%, 1.9 ± 0.3% and 1.5 ± 0.1% respectively.

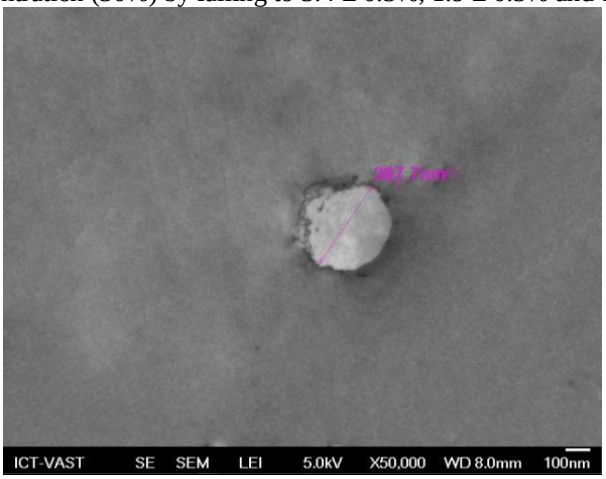

Figure 3. Representative SEM images showing the isolated minicells of *Leuconostoc* with the nanopartile size determination.

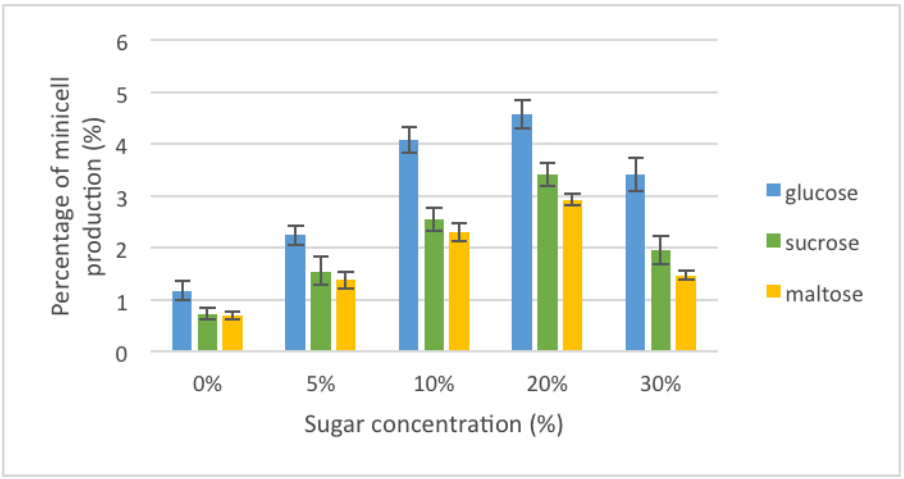

Figure 4. The percentage of minicell production from *Leuconostoc* in modified Lactobacilli MRS medium with differently selected sugars, as glucose, sucrose and maltose, with different concentrations (0%, 5%, 10%, 20% and 30% w/v).

Hence, this glucose medium (20% glucose w/v) was utilized as appropriate condition in order to produce minicell for study of drug delivery.

3.3. Antimicrobial Activity Of Minicells

To test the packaging ability of *Leuconostoc* minicells, agar diffusion test was done. Finally, activities of minicells were shown (Table 2).

Table 2. Antimicrobial activity of silver-packaged minicells of *Leuconostoc* against *Pseudomonas aeruginosa*

Groups	Zone of inhibition (mm)
Minicell	14.3 ± 1.2
Minicell- Ag+	19.3 ± 0.8
Ag+	24.5 ± 0.5

Data was reported as means ± standard deviations

The obtained results in the experiments presented that minicells had abilities to be packaged by metal ions, especially the silver ions in this case. The figure 5 clearly that all of solution possessed potency of antimicrobial activity, including isolated minicells, silver-packaged minicells, and 1mM silver nitrate solution with the inhibition zone diameters by 14.3±1.2 mm, 19.3±0.8mm, and 24.5±0.5mm respectively. The phosphate buffer saline (PBS) solution was utilized as a negative control that had no antimicrobial activities. The statistical analysis results also presented that there were significant different in their antimicrobial ability when isolated minicells from *Leuconostoc* added with silver nitrate

solution for 20hrs ($p<0.05$). Based on the inhibition zone diameter, minicells could package with silver. By calculation of diameter and amount of of silver solution, minicells ($6x10^5$) could package about 0.069 µg $AgNO_3$. The potency of minicells ($6x10^5$) equaled to about 0.85 µg $AgNO_3$. Consequently, minicells could be used as nanoparticles in antimicrobial activity or drug delivery.

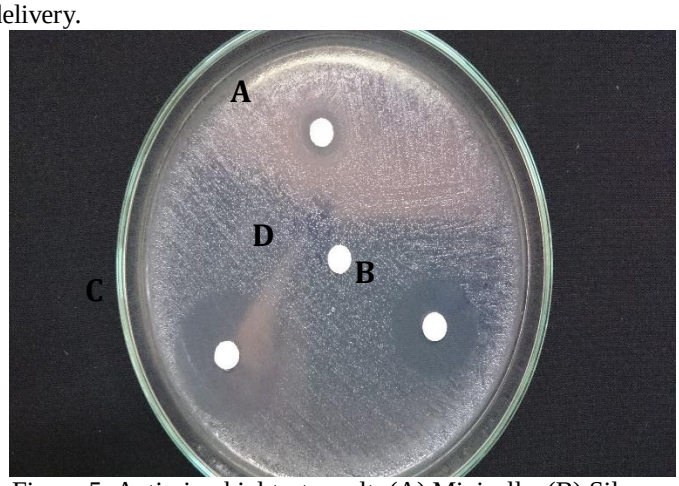

Figure 5. Antimicrobial test result: (A) Minicells; (B) Silver-packaged minicells; (C) 1mM Silver solution; (D) PBS buffer as negative control

4. Conclusion

This is the first study on minicell production by *Leuconostoc*. Although the time and working conditions were limited, the study optimized four kinds of sugars (glucose, sucrose, and maltose) in different concentration (0%, 5%, 10%, 20%, and 30%). The results suggested the method to produce minicells (400 nm) that could be a source of nanoparticle in the inhibition on *Pseudomonas aeruginosa*. Secondly, minicells could package to silver. Therefore, these minicells could be also used in drug delivery applied in pharmaceutical field.

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