Hindawi
Journal of Nanomaterials
Volume 2019, Article ID 7684795, 9 pages
https://doi.org/10.1155/2019/7684795

Research Article

Tinidazole Delivery Improved by Nanosized Minicells Originated from *Leuconostoc mesenteroides*

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Received 24 July 2018; Revised 10 December 2018; Accepted 12 December 2018; Published 10 February 2019

Academic Editor: Renyun Zhang

A nanoparticle originating from natural products used as a drug delivery is considered as one of the indispensable issues in the pharmaceutical field. Increasing the bioavailability of a drug and prolonging the effect of the drug are important. Tinidazole is an antifungal agent that has absorption interfered by food. This study reported the ability of *Leuconostoc mesenteroides* VTCC B-871 in producing nanosized minicells used as drug delivery for tinidazole to improve the passage to the eaten mouse intestinal membrane. By using a scanning electron microscope and a transmission electron microscope, the morphology of the minicell loading drug was observed. The spherical shape and size (400 nm) of minicells did not change over time when kept in buffered saline gelatin and packaged with tinidazole. Based on Box-Behnken design, the optimal conditions were selected for actual encapsulation. Minicells could encapsulate tinidazole approximately to 90% which was determined by high-performance liquid chromatography analysis. The maximal concentration of tinidazole released from minicells was 70% at pH 3.4 and 55% at pH 7.2, respectively. The absorption ability of tinidazole packaging minicells was quantified in mice. Tinidazole loading minicells could be absorbed faster than tinidazole alone in fed mice via oral administration. The study assessed that the absorption of water-insoluble tinidazole could be improved by *Leuconostoc* minicells without inhibition by food effects.

1. Introduction

Currently, developing a novel type of drug delivery is a very important goal in pharmaceutical research, especially nanoparticle drug delivery. The therapeutic agent can be packaged, dispersed, or encapsulated with a nanoparticle which acts as a drug delivery for directing the drug to specific targets by recognizing a specific ligand on their surface, increasing the drug's penetrating efficiency and drug half-life or also manipulating circulation time and bioavailability by changing the nanoparticle size and surface characteristics of the nanoparticle [1]. It is worth noting that these abilities are not only the improvement of drug absorption, which can lead to the reduction of drug dosing interval thereby decreasing toxicity, but also the advances in therapeutic efficacy by increasing the target precession as well. Nanoparticles can be nanopowder, nanocluster, or nanocrystal [2]. For pharmaceutical technology, a nanoparticle used as drug delivery is defined as a submicron whose range is less than 1 μm. The devices or materials are of different varieties including polymers, lipids (liposomes), magnetic, even inorganic or metallic compounds (iron, silica), and bacteria (bacterial nanoparticles or "minicells"). Normally, the drug releases nanoparticles by diffusion, erosion, swelling, and degradation after entering the body. However, there has been little success due to several reasons: low drug loading in the cell carrier, untimely drug release instead of continuous unloading over time, and limited drug administration because of its characteristics. As all of these problems had been reported in many nanoparticles (e.g., liposomes, micelles, nanospheres, and nanofibers) [1], finding an ideal drug delivery with an optimal size, shape, and surface

characteristic including the specific target binding size is still a challenge for scientists. The minicell is described as a type of abnormal division in bacteria, which came from a mutation or stress environment [3], resulting in a small spherical bacterium with a diameter less than 1 μm. Scientists suggested that this was a new term for "nanocells" due to the fact that it has its diameter at the scale of nanometers. Even though the minicell has a disrupted cell cycle and contains no chromosomal DNA, it still retains most of the components from the parents including lipopolysaccharides, cell wall, normal envelop structures, transport system, and the ability to transcribe or translate the genes of recombinant plasmid if the plasmid is present in minicells. Therefore, minicells could be a good carrier candidate. At present, there are some strains of bacteria reported to possibly induce a form of minicell [4, 5] such as *Bacillus subtilis* [6], *Salmonella typhimurium* [7], *Haemophilus influenzae* [8], *Shigella flexneri* [9], *Pseudomonas aeruginosa* [10], and *Listeria monocytogenes* [11]. Up to date, using intact bacterial minicells for drug delivery, which can distribute the drug into a specific target both *in vivo* and *in vitro* which are considered as a novel delivery vehicle when they are water-soluble and have biocompatible properties, is one of the solutions which will be a driving force in nanoparticle technology in the 21st century in finding an ideal drug delivery [4, 5].

Probiotics are living microorganisms which contribute health benefits to the host [12]. Some bacterial strain examples included *Lactobacillus*, *Leuconostoc*, and *Bifidobacterium*. Moreover, many researches have indicated that the probiotic effect is strain-specific, which means the bacteria only express the effect in specific cases triggered by their exposure to particular characteristics such as resistance to gastric acid and bile or the ability to colonize the mucosa and antimicrobial activity [13].

The genus *Leuconostoc* belongs to the family of *Leuconostocaceae*. *Leuconostocs* are closely related to *Fructobacillus*, *Weissella*, and *Oenococcus*. Together, they are commonly known as the "*Leuconostoc* group" of lactic acid bacteria (LAB). Besides improving biological functions of the host, the LAB group also plays an important role in helping people who suffer a tumor and immune compromised subjects. According to Bergey's Manual of Systematic Bacteriology, *Leuconostoc* is a Gram-positive bacterium that has small cells and its regular, generally ovoid *cocci* are linked to form short chains. The other characteristics include nonmotility and no spore formation, but they are acid-loving. Last but not least, they would not uncommonly cause a disease to humans and also affect the immune system by acting the immune cells via different mechanism signals [14]. Based on these properties, *Leuconostoc mesenteroides* was studied for drug delivery systems [15]. Tinidazole, which mainly contains 5-nitroimidazoles, is an analogue structure of metronidazole, a popular antifungal agent for its effectiveness and acceptable tolerability. In 1991, a high level of metronidazole resistance was reported as 1 in over 2000-3000 cases, especially in vaginal trichomoniasis. Tinidazole is poorly dissolved in water (1.99×10^{-4} mg/l). Administration of tinidazole with food reduces by 10% of C_{max} [16]. These problems led to a need for the development of tinidazole formulation for higher effects in case of food presence. Therefore, development of tinidazole formulation should be improved. In this study, tinidazole should be packaged in a drug delivery system. In order to find out the best condition for packaging study or drug encapsulation efficiency, Box-Behnken which was an experimental design [17] was used for analyzing multivariate experiments [18]. We carried out an assessment about *Leuconostoc mesenteroides* minicells in encapsulation and release of tinidazole and *in vivo* evaluation of permeation ability across the small intestine in the combination of food. This is the first *in vivo* study of *Leuconostoc mesenteroides* on insoluble tinidazole delivery in the combination of food. The successful study will be applied for improving the absorption of many insoluble drugs interfered with food so far.

Table 1: The levels of variables chosen for the trials.

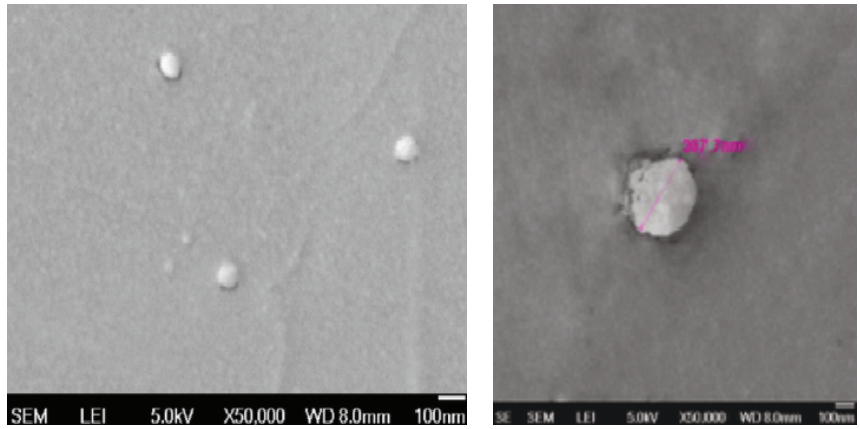
Volume of minicells (μl)	Tinidazole (mg/ml)	Time (h)
10 (-1)	0.1 (-1)	4 (-1)
55 (0)	0.5 (0)	10 (0)
100 (1)	0.9 (1)	16 (1)

Table 2: The matrix of variables chosen for the trials.

Trial	Volume of minicells (μl)	Tinidazole (mg/ml)	Time (h)
1	10	0.1	10
2	10	0.9	10
3	100	0.1	10
4	100	0.9	10
5	10	0.5	4
6	10	0.5	16
7	100	0.5	4
8	100	0.5	16
9	55	0.1	4
10	55	0.1	16
11	55	0.9	4
12	55	0.9	16
13	55	0.5	10
14	55	0.5	10
15	55	0.5	10

2. Materials and Methods

Leuconostoc mesenteroides VTCC-B-871 was purchased from the Vietnam Type Culture Collection (Vietnam). *Lactobacilli* MRS broth, D-glucose, acetonitrile, gelatin, formaldehyde, and sodium chloride were from Merck (Germany). Standard tinidazole was obtained from the Institute for Drug Quality Control, Ministry of Health (Vietnam). The 0.45 μm filter membrane (Sigma, USA), dialysis tube (Sigma, USA), centrifugator (Kubota, Japan), optical microscope (Labomed, USA), scanning electron microscope (Hitachi S-4800, Japan), transmission electron microscope (JEOL JEM-1400), microplate reader (Biotek, USA), high-performance liquid



(a) (b)

Figure 1: The morphology of minicell representatives observed under SEM. (a) 50000x magnification; (b) 100000x magnification.

chromatography system including a C18-column and DAD detector (Shimadzu, Japan), and pH meter (Schott, Germany) were used in the study.

2.1. Leuconostoc Minicell Preparation. This bacterium was incubated in modified *Lactobacilli* MRS broth with 20% D-glucose for 48 hours at room temperature. The culture was collected and checked to ensure minicell formation before isolation according to our previous study [18].

2.2. Minicell Stability Test. First, the minicell fraction must be separated with their parent bacterial cells and cell debris for guaranteeing their purification. The culture was centrifuged at 3500 g for 20 min to remove the large cells. After the supernatant had been collected, it was filtered through a 0.45 μm filter membrane twice to completely separate minicells from cell debris and their parental cells. Then, the collected supernatant was centrifuged at 15000 g in 20 min. The collected pellets were minicells. Minicells were then resuspended in 1x buffered saline gelatin (BSG) solution and kept for later use.

2.3. Microscopic Test. The isolated minicells were observed by a light microscope at 100x magnification for counting the number of minicells and examining their morphology. The number of minicells was calculated as the following equation: the density of obtained minicells (cell/ml) = (number of cells × 10 000)/number of squares.

2.4. Scanning Electron Microscopy (SEM). In order to determine the size of minicells, SEM was used. Before scanning, minicells were mixed with 4% formaldehyde in phosphate-buffered saline (PBS) solution and incubated overnight at room temperature. After that, minicells were collected and washed with PBS solution to completely remove 4% formaldehyde. Minicells went through dehydration, respectively, with 30, 50, 70, 80, 90, and 100%. Each dehydration step took 10 min for incubation.

2.5. Design for the Optimization of the Best Condition for Packaging Minicells with Tinidazole. The optimization is based on the Box-Behnken experimental design. Tables 1 and 2 show the design for tinidazole-incubated minicell in different times. All 15 cases were carried out for setting up the Box-Behnken program. The system involved 3 independent factors (volume of tinidazole (X_1), tinidazole concentration (X_2), and time for incubation (X_3)). The mathematical relationship between these factors was approximately quantified by this quadratic equation:

$$Y = C_0 + C_1X_1 + C_2X_2 + C_3X_3 + C_{12}X_1X_2 + C_{13}X_1X_3 + C_{23}X_2X_3 + C_{11}X_1^2 + C_{22}X_2^2 + C_{33}X_3^2. \quad (1)$$

Y = predicted yield; C_0 = constant response; C_1, C_2, and C_3 = linear coefficients; C_{12}, C_{13}, and C_{23} = cross product coefficients; and C_{11}, C_{22}, and C_{33} = quadratic coefficients.

2.6. Drug Encapsulation Efficiency (EE). Based on the design, the combination between tinidazole and *Leuconostoc* minicells

Table 3: Experimental and theoretically predicted values for concentration of tinidazole-minicells.

Experimental no.	Actual value (mg/ml)	Predicted value (mg/ml)
1	0.0992	0.0989
2	0.8996	0.8997
3	0.0998	0.0996
4	0.8994	0.8997
5	0.4999	0.4999
6	0.4995	0.4995
7	0.4999	0.4999
8	0.4999	0.4999
9	0.0996	0.0997
10	0.0996	0.0999
11	0.8998	0.8996
12	0.8994	0.8993
13	0.4987	0.4988
14	0.4987	0.4989
15	0.4987	0.4985

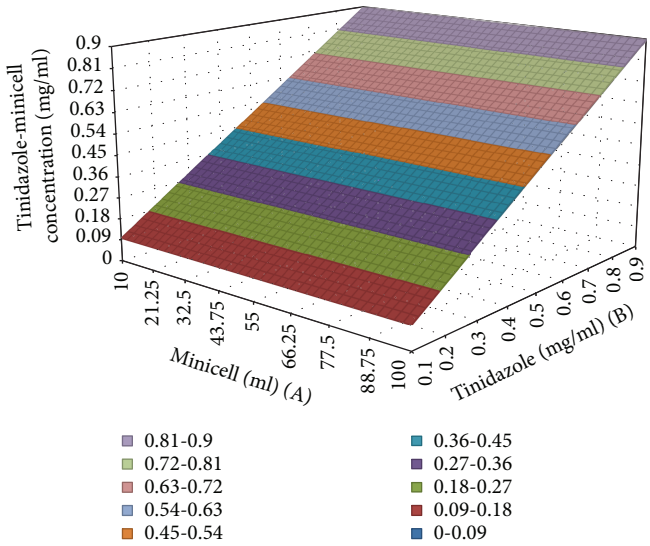


Figure 2: Surface plot representing concentration of tinidazole-minicell obtained from actual volume of minicells (a) and tinidazole concentration (b).

in different conditions was done. The mixtures were collected by centrifugation at 15,000 g within 30 min in 4°C. The supernatant was used to quantify remaining tinidazole based on the optical density measurement. Then, tinidazole concentration in minicells was determined by subtracting the concentration of initial tinidazole with the remaining quantified tinidazole. The drug encapsulation efficiency (EE) was calculated by the following equation:

$$\text{Encapsulation efficiency} = \frac{\text{drug concentration in minicells} \times 100}{\text{total drug concentration}}. \quad (2)$$

2.7. In Vitro Dissolution Test. A dialysis tube was applied for nanoparticle dissolution test [19]. Dissolution of minicell loading tinidazole was performed in stomach condition-like fluid (pH 3.4) and plasma-like fluid (pH 7.2) during 24 h. Supernatants were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 12, and 24 h. The drug release levels were measured, and the peak area was analyzed by HPLC and spectrophotometry as measuring the absorbance using a multimode microplate reader.

2.8. Absorption of Tinidazole Minicells. Before mice were administered with tinidazole-minicell and tinidazole (400 μg, a single 50 mg/kg dose), mice had been fed normally and their health observed for 1 month. After this period, mice were orally administered with tinidazole-minicell and tinidazole in fed mice. Every 1 h, their blood samples were taken for determining the presence of tinidazole. The experiment was performed during 5 h.

2.9. Analyzing Drug Concentration in Blood. Mice were held to have their blood taken in the tail. The blood was then mixed with acetone, and a vortex at 2000 g was performed. The precipitation of blood cells was collected by a 5000 g centrifugation step in 20 min at 4°C. The supernatant containing tinidazole was taken. Tinidazole concentration was then measured using high-performance liquid chromatography (HPLC).

2.10. High-Performance Liquid Chromatography. The mobile phase contained the main components which are acetonitrile, methanol, and phosphate buffer [20]. The flow rate was at 1 ml/min on a C18 column. The UV/Vis detector was set at 320 nm. The limit of detection was 0.05 μg/ml.

2.11. Antifungal Activities. In order to confirm tinidazole in serum, sera collected in 1, 2, 3, 4, and 5 h in fed mice with tinidazole/minicell-tinidazole were used to test on *Trichomonas*. The method was agar dilution test. Standard tinidazole (0.9 mg/ml) was used as positive control. The negative control was the serum collected from mice without tinidazole administration. The positive control, negative control, and all collected sera were then added in the semisolid agar for yeast mold (Cat No. 1194, Conda, Spain). One inoculum of fungi was prepared with turbidity equaling to the 0.5 McFarland standard. 1 μl of the inoculum suspension was dropped into the medium with collected sera and standard tinidazole. All cultures were incubated at 35°C until stable growth in medium without tinidazole. Colony count was done in different media. The inhibition percentage of tinidazole was calculated based on the ratio of survival colonies grown in media added with collected sera and standard tinidazole.

2.12. Data Analysis. The results of triplicate for all experiments were stated as mean ± standard deviation (SD) and then analyzed by one-tailed t-test and two tailed t-test for paired comparison of means. The statically significant differences were considered with $P < 0.05$.

Table 4: The representative encapsulation quantum obtained in the surface plot.

Tinidazole concentration (mg/ml)	Volume of minicell (/ml)												
	10	12.25	14.5	16.75	19	21.25	23.5	25.75	28	30.25	32.5	34.75	37
0.1	0.099164496	0.09913247	0.099102822	0.099075553	0.099050663	0.099028151	0.099008018	0.098990264	0.098974888	0.098961891	0.098951272	0.098943032	0.098937171
0.12	0.119144695	0.119112271	0.119082226	0.119054559	0.119029271	0.119006362	0.118985831	0.118967679	0.118951905	0.11893851	0.118927494	0.118918856	0.118912597
0.14	0.139126341	0.139093519	0.139063076	0.139035012	0.139009326	0.138986019	0.13896509	0.13894654	0.138930368	0.138916576	0.138905162	0.138896126	0.138889469
0.16	0.159109432	0.159076213	0.159045372	0.15901691	0.158990826	0.158967121	0.158945795	0.158926847	0.158910278	0.158896087	0.158884275	0.158874842	0.158867787
0.18	0.17909397	0.179060353	0.179029114	0.179000254	0.178973773	0.17894967	0.178927946	0.178908601	0.178891634	0.178877045	0.178864836	0.178855004	0.178847552
0.2	0.199079954	0.199045939	0.199014303	0.198985045	0.198958166	0.198933665	0.198911543	0.1988918	0.198874435	0.198859449	0.198846842	0.198836613	0.198828763
0.22	0.219067384	0.219032972	0.219000937	0.218971282	0.218944005	0.218919107	0.218896587	0.218876446	0.218858683	0.2188433	0.218830294	0.218819668	0.21881142
0.24	0.239056261	0.23902145	0.238989018	0.238958965	0.23893129	0.238905994	0.238883077	0.238862538	0.238844377	0.238828596	0.238815193	0.238804168	0.238795523
0.26	0.259046583	0.259011375	0.258978545	0.258948094	0.258920022	0.258894328	0.258871012	0.258850076	0.258831518	0.258815338	0.258801538	0.258790115	0.258781072
0.28	0.279038352	0.279002746	0.278969518	0.278938669	0.278910199	0.278884107	0.278860394	0.27883906	0.278820104	0.278803527	0.278789328	0.278777508	0.278768067
0.3	0.299031566	0.298995563	0.298961937	0.298930691	0.298901823	0.298875333	0.298851223	0.29882949	0.298810137	0.298793162	0.298778566	0.298766348	0.298756509
0.32	0.319026227	0.318989826	0.318955803	0.318924158	0.318894893	0.318868005	0.318843497	0.318821367	0.318801616	0.318784243	0.318769249	0.318756633	0.318746396
0.34	0.339022334	0.338985535	0.338951114	0.338919072	0.338889409	0.338862124	0.338837217	0.33881469	0.33879454	0.33877677	0.338761378	0.338748365	0.33873773
0.36	0.359019888	0.35898269	0.358947872	0.358915432	0.358885371	0.358857688	0.358832384	0.358809458	0.358788912	0.358770743	0.358754954	0.358741543	0.35873051
0.38	0.379018887	0.378981292	0.378946076	0.378913238	0.378882779	0.378854699	0.378828997	0.378805673	0.378784729	0.378766163	0.378749975	0.378736167	0.378724736
0.4	0.399019333	0.39898134	0.398945726	0.39891249	0.398881634	0.398853155	0.398827056	0.398803335	0.398781992	0.398763028	0.398746443	0.398732237	0.398720409
0.42	0.419021224	0.418982834	0.418946822	0.418913189	0.418881934	0.418853058	0.418826561	0.418802442	0.418780702	0.41876134	0.418744357	0.418729753	0.418717527
0.44	0.439024562	0.438985774	0.438949364	0.438915333	0.438883681	0.438854407	0.438827512	0.438802995	0.438780857	0.438761098	0.438743717	0.438728715	0.438716092
0.46	0.459029346	0.45899016	0.458953353	0.458918924	0.458886874	0.458857202	0.458829909	0.458804995	0.458782459	0.458762302	0.458744524	0.458729124	0.458716103
0.48	0.479035576	0.478995993	0.478958788	0.478923961	0.478891513	0.478861444	0.478833753	0.478808441	0.478785507	0.478764952	0.478746776	0.478730979	0.478717559
0.5	0.499043253	0.499003271	0.498965668	0.498930444	0.498897598	0.498867131	0.498839043	0.498813333	0.498790002	0.498769049	0.498750475	0.498734279	0.498720463
0.52	0.519052375	0.519011996	0.518973995	0.518938373	0.51890513	0.518874265	0.518845779	0.518819671	0.518795942	0.518774591	0.51875562	0.518739026	0.518724812
0.54	0.539062944	0.539022167	0.538983768	0.538947749	0.538914107	0.538882845	0.538853961	0.538827455	0.538803328	0.53878158	0.538762211	0.53874522	0.538730607
0.56	0.559074959	0.559033784	0.558994988	0.55895857	0.558924531	0.558892871	0.558863589	0.558836686	0.558812161	0.558790015	0.558770248	0.558752859	0.558737849
0.58	0.57908842	0.579046847	0.579007653	0.578970838	0.578936401	0.578904343	0.578874663	0.578847362	0.57882244	0.578799896	0.578779731	0.578761945	0.578746537
0.6	0.599103327	0.599061357	0.599021765	0.598984552	0.598949717	0.598917261	0.598887184	0.598859485	0.598834165	0.598811223	0.598790661	0.598772476	0.598756671
0.62	0.61911968	0.619077312	0.619037323	0.618999712	0.618964479	0.618931626	0.618901151	0.618873054	0.618847336	0.618823997	0.618803036	0.618784454	0.618768251
0.64	0.63913748	0.639094714	0.639054327	0.639016318	0.638980688	0.638947436	0.638916563	0.638888069	0.638861953	0.638838216	0.638816858	0.638797878	0.638781277
0.66	0.659156725	0.659113562	0.659072777	0.65903437	0.658998342	0.658964693	0.658933422	0.65890453	0.658878017	0.658853882	0.658832126	0.658812748	0.658795749
0.68	0.679177417	0.679133856	0.679092673	0.679053869	0.679017443	0.678983396	0.678951728	0.678922438	0.678895527	0.678870994	0.67884884	0.678829065	0.678811668
0.7	0.699199555	0.699155596	0.699114015	0.699074813	0.69903799	0.699003545	0.698971479	0.698941791	0.698914482	0.698889552	0.698867	0.698846827	0.698829032
0.72	0.719223139	0.719178782	0.719136804	0.719097204	0.719059983	0.71902514	0.718992676	0.718962591	0.718934884	0.718909556	0.718886607	0.718866036	0.718847843
0.74	0.73924817	0.739203415	0.739161039	0.739121041	0.739083422	0.739048182	0.73901532	0.738984837	0.738956732	0.738931006	0.738907659	0.73888669	0.7388681
0.76	0.759274646	0.759229494	0.75918672	0.759146324	0.759108307	0.759072669	0.75903941	0.759008529	0.758980027	0.758953903	0.758930158	0.758908791	0.758889804
0.78	0.779302569	0.779257018	0.779213847	0.779173054	0.779134639	0.779098603	0.779064946	0.779033667	0.779004767	0.778978246	0.778954103	0.778932339	0.778912953

Table 4: Continued.

Tinidazole concentration (mg/ml)	Volume of minicell (/ml)												
	10	12.25	14.5	16.75	19	21.25	23.5	25.75	28	30.25	32.5	34.75	37
0.8	0.799331937	0.799285989	0.79924242	0.799201229	0.799162417	0.799125983	0.799091928	0.799060252	0.799030954	0.799004034	0.798979494	0.798957332	0.798937548
0.82	0.819362752	0.819316406	0.819272439	0.819230851	0.819191641	0.819154809	0.819120356	0.819088282	0.819058586	0.819031269	0.819006331	0.818983771	0.81896359
0.84	0.839395013	0.83934827	0.839303905	0.839261918	0.839222311	0.839185081	0.839150231	0.839117759	0.839087665	0.839059951	0.839034614	0.839011657	0.838991078
0.86	0.859428721	0.859381579	0.859336816	0.859294432	0.859254427	0.8592168	0.859181551	0.859148682	0.85911819	0.859090078	0.859064344	0.859040989	0.859020012
0.88	0.879463874	0.879416335	0.879371174	0.879328392	0.879287989	0.879249964	0.879214318	0.879181051	0.879150162	0.879121651	0.87909552	0.879071767	0.879050392
0.9	0.899500474	0.899452537	0.899406978	0.899363799	0.899322997	0.899284575	0.899248531	0.899214866	0.899183579	0.899154671	0.899128141	0.899103991	0.899082218

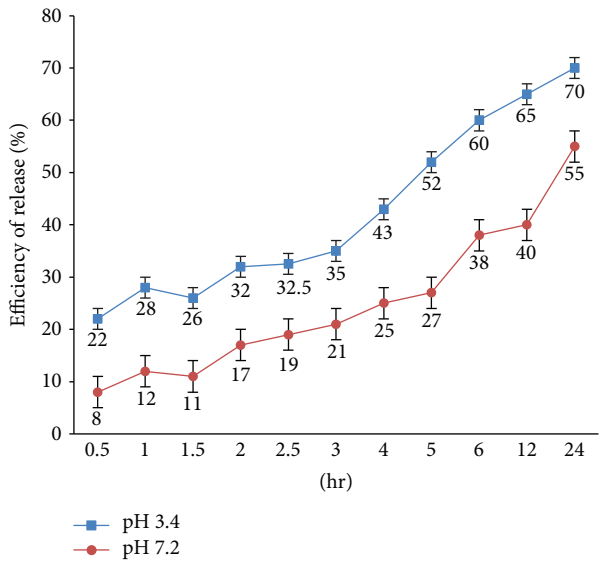


Figure 3: Tinidazole released from minicells in different pH.

3. Results and Discussions

The *Leuconostoc* minicell diameter was less than 400 nm and kept in a BSG solution for 1 month as well as packaged with tinidazole. Figure 1 shows the representatives of minicells in nanosize in different incubations when they were observed under SEM. From these results, it was meant that minicells were stable for a time and not aggregative which could be suggested as vehicle for drug delivery. As seen in Figure 1, *Leuconostoc* minicells were stable for a time when the component of the outer layer of the bacterium changed due to the sugar stress after being cultured in different sugars [21]. Moreover, there was a connection of cell division inhibitor proteins, like FtsZ and MinD, with sugars, leading to cell differentiation of lactic acid bacteria [22].

According to the different concentration levels of tinidazole packaged with minicells (tinidazole-minicell), which was generated at different volumes of minicell, tinidazole's concentration, and time for incubation, the regression equation was formed [23].

$Y = 0.4987 + 0.0001X_1 + 0.4000X_2 - 0.0001X_3 - 0.0002X_1X_2 + 0.0001X_1X_3 - 0.0001X_2X_3 + 0.0005{X_1}^2 + 0.0003{X_2}^2 + 0.0006{X_3}^2$, where Y is the concentration of tinidazole-minicell. The quadratic regression was significant at the level of 95%. The conditions for packaging tinidazole with minicells were based on this equation. From this condition matrix, the predicted and experimental results of minicells in packaging with tinidazole are summarized in Table 3.

The high fitness between predicted and actual values of response Y showed that the model was valid for designation of optimal conditions for minicell packaging with tinidazole taking time barely for optimization. Table 2 expresses the design matrix including 3 factors (time of incubation, volume of minicells, and tinidazole concentration) in 15 runs and 3 center points. Basing on the design in Table 2, the actual encapsulation was fit to predicted encapsulation (Table 3), showing that the Box-Behnken design was available in the study. As shown in Table 3, drug encapsulation efficiency (EE) could be obtained in conditions (2, 4, 11, and 12) at approximately 90. The potential validation points of the A factor (minicells) were 32.5 and 77.5 ml. Validation points for the B factor (tinidazole) were 0.3 and 0.7 mg/ml. Validation points for incubation time were 7 and 13 hours. To visualize the correlation of tinidazole concentration and volume of minicells, a surface or contour plot was created. The design was selected for constant incubation time during 10 hours to see the encapsulation quantum of tinidazole in minicells when changing the volume of minicells and tinidazole concentration that was expressed in a surface plot (Figure 2). Table 4 gives the representative encapsulation quantum recorded in a surface plot. From four conditions (Table 2) giving the maximal encapsulation (Table 3) and analysis as shown in Figure 2 and Table 4; the final condition used for encapsulation, drug release test, and *in vivo* absorption was condition 2 including time of incubation (10 hour), volume of minicells (10 ml), and tinidazole concentration (0.9 mg/ml). In the other output (Table 3), the volume of minicells was too high while tinidazole concentration was too low and vice versa. Therefore, the encapsulation efficacy was not ideal as other conditions mentioned in Table 2. Tinidazole could be packaged and released out of minicells that, probably, oxygen of nitro moiety of tinidazole interacted with hydrogen of the hydroxyl group of sugar monomers. Therefore, the drug packaging time should be optimized to get a high efficacy. The results also showed more clarification of the results in Figure 3. The release started after 2 h in testing pH media and was obtained highly at 24 h. Tinidazole released from minicells in basic medium was slower than that in acidic medium. Probably, the acidic medium could be involved in hydrolyzation of the bond of minicells and tinidazole.

Tinidazole should be released out of minicells in suitable pH conditions to show bioavailability without effects of minicells when administered orally. Therefore, the media at pH 3.4 and 7.2 were used for the dissolution test (Figure 3). The maximal mean concentration of tinidazole released from minicells was 70% at pH 3.4 and 55% at pH 7.2 after 24 h, respectively. The release started after 2 h in testing pH media and was obtained highly at 24 h. Tinidazole released from minicells in basic medium was slower than in acidic medium. Probably, the acidic medium could be involved in the hydrolyzation of the bond of minicells and tinidazole. Besides, there was a significant difference in release efficiency of tinidazole loaded by minicells between pH 3.4 and pH 7.2 (Figure 3).

By high-performance liquid chromatography (HPLC) analysis, the retention time of the peak appearing in the chromatogram of the blood sample (3.5265 min) was similar to standard tinidazole (3.5395 min), suggesting tinidazole's existence in blood (Figure 4). Interestingly, there was also a high chromatographic peak at 3.126 min (Figure 4(b)). Probably, this peak expressed the existence of metabolite of tinidazole when minicell-tinidazole entered the bloodstream. A similar phenomenon was also obtained when tinidazole entered the bloodstream alone

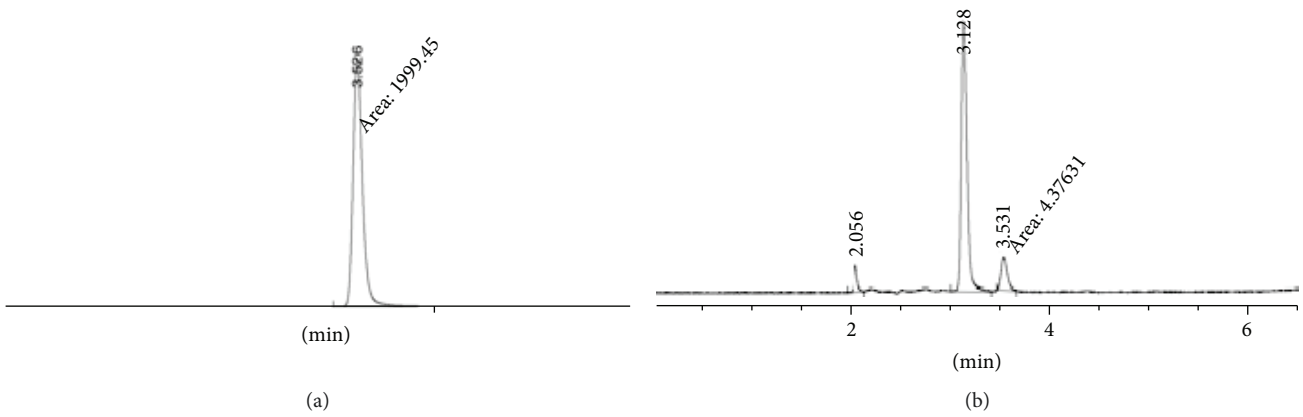


Figure 4: HPLC of standard tinidazole (a) and tinidazole in serum (b).

Table 5: Concentration of tinidazole and tinidazole-minicells in serum.

Drugs	Concentration (mg/ml)					
	0 h	1 h	2 h	3 h	4 h	5 h
Tinidazole	0	0.29 ± 0.59	1.3133 ± 0.9	1.09 ± 0.85	0.54 ± 0.73	0.32 ± 0.27
Tinidazole delivered by minicells	0	2.31 ± 0.63	8.1567 ± 2.4	7.19 ± 1.2	6.39 ± 0.48	3.17 ± 0.94

Table 6: Anti-trichomonas ability of tinidazole and tinidazole-minicells collected from serum.

Drugs	Inhibition percentage (%)					
	0 h	1 h	2 h	3 h	4 h	5 h
Tinidazole	0%	1 ± 0.5	6 ± 0.41	6 ± 0.28	3 ± 0.2	1 ± 0.64
Tinidazole delivered by minicells	0%	10 ± 1.3	40 ± 1.12	35 ± 0.56	30 ± 0.37	17 ± 0.09

as well. Moreover, tinidazole-minicells could be absorbed into the blood to give a higher tinidazole than using tinidazole alone in fed mice. The existence of tinidazole in serum was determined every 1 h during 5 h (Table 5). After 2 h absorption, tinidazole-minicells in serum was 8.1567 ± 2.4007 μg while tinidazole in blood was 1.3133 ± 0.9059 μg. Tinidazole-minicells gave tinidazole absorption at the highest maximal concentration after 2 h absorption in fed mice. Tinidazole-minicells could be absorbed faster and obtained 8 times higher than tinidazole alone in fed mice. After 5 h, tinidazole concentration in both cases was reduced (Figure 4). However, the tinidazole concentration in minicell formulation always gave a higher amount than did tinidazole used alone. Tinidazole from minicells was determined in blood more highly than was tinidazole used alone in eaten mice. It was meant that *Leuconostoc* minicells had the cell structure homogenizing with the intestinal tract membrane as a phospholipid through which tinidazole-minicells could pass easily. The study suggested that tinidazole should be delivered by *Leuconostoc* minicells. With this delivery system, food did not interfere with the absorption of tinidazole as informed before.

Additionally, by testing the anti-*Trichomonas* activity of tinidazole after being administered in fed mice, the collected serum showed inhibition on *Trichomonas* (Table 6) that suggested tinidazole's existence in serum. However, tinidazole-minicell showed higher activities than did tinidazole used in fed mice when comparing with the inhibition activity of standard tinidazole carried out *in vitro*. To know whether sera are involved in the activity, sera obtained from mice without using any kind of tinidazole were also tested and did not show inhibition on fungus. Obviously, tinidazole delivered by minicells could release tinidazole in blood and still show antifungal activity.

To sum up, *Leuconostoc* could be used as a drug delivery for tinidazole when differentiating this bacterium into minicells in different sugars. The delivery ability of these minicells seemed similar as minicells originated from mutant bacteria, like *Bacillus* [24]. Minicells prepared in the study were safer when there was no effect caused by genetic engineering. This study contributes an alternative delivery system for poorly water-soluble compound absorption interfered with food besides polymeric micelles [25].

Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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