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Microcalorimetric studies of the effects of artesunate liposomes on the metabolism of *Escherichia coli* during growth

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Abstract A thermal dynamic model of nanoformulations entrapped in artesunate liposomes was established and biological thermodynamics was applied for investigation of the drug formulations. Effects of artesunate liposomes on the growth metabolism of *Escherichia coli* were studied by microcalorimetry. The results showed that (1) Comparison of artesunate and artesunate liposomes, the thermogenesis curves of *E. coli* were significant different in the metabolic process: lag phase (AB), log phase (BC), stationary phase (CD), and decline phase (DE); (2) Linear fit of the data of total metabolic heat of *E. coli* effected by different concentration artesunate (1–300 μg), the equation can be obtained as follows: $Y = 364720.61 - 1075.25x$, $R = 0.9985$; Linear fit of the data of total metabolic heat of *E. coli* effected by different concentration artesunate liposomes (30–120 μg), the linear equation can be obtained as follows: $Y = 54251.5765 - 35.71122x$, $R = 0.98345$; (3) The half inhibitory concentration I_{C50} was 50.05 μg/mL, the relative sensitivity was obviously different; (4) Artesunate liposomes having better sustained release properties as compare to artesunate.

Keywords Microcalorimetry · Artesunate liposomes · *Escherichia coli* · Thermokinetics

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Introduction

Artemisinin derivative is a broad-spectrum drug with a complete new anti-malarial mechanism, of which China owns the unique independent intellectual property rights [1]. It has offered a drug with high efficacy and low toxicity in the treatment of chloroquine-resistant, multi-drug-resistant cerebral malaria, and falciparum malaria [2]. Artemisinin is a promising and potent anti-malarial drug, which meets the dual challenges posed by drug-resistant parasites and rapid progression of malarial illness [3–5]. Along with the development of research, it is found that artemisinin-based compounds have many other important pharmacological functions such as anti-malarial, tumor cell toxicity, immune regulation, anti-progesterone, anti-schistosome, anti-toxoplasma, anti-arrhythmic, anti-fiber role, and so on [4–6]. In this project, the extent and duration of the inhibitory effect on the metabolism of *E. coli* were judged from the rate constant k [5]. It provides much kinetic and thermodynamic information that cannot be obtained by conventional bacteriological techniques, and all this information is very significant for research of the metabolism in organisms and the mechanism of thermodynamic model of the artemisinin-based drugs [6–8].

Escherichia coli is a common bacterium that is normally present in the human intestine [9, 10]. There are different types, or strains, of *E. coli* that infect many types of animals. Commonly the types of intestinal bacteria of usual residence in human and animals do not cause diseases, but the bacteria can cause extra-intestinal infections under certain conditions [11]. Some serotypes bacteria with highly pathogenic, causing diarrhea, which are called pathogenic *E. coli*. In present, a lot of studies have proved that *E. coli* is an ideal model for evaluations of drugs, toxic effects, organic, and inorganic substances and water pollutants [12, 13].

Nanotechnology is an emerging technology (nano-sized 10^{-9}–10^{-7} m) [14, 15]. The rearrangements of atoms and molecules of the same or different elements in physical space result in one or more new substances created. Owning to the amazing changes occured in the physical and chemical properties and the biological characteristics, applications of the new substances have become the frontiers of science in new century in the pharmaceutical industry [16]. Nanotechnology has been used in development of modern pharmaceutical preparations, to make drugs with stability, low toxic side effects, gastrointestinal irritation, high drug utilization, and many other advantages. Therefore, the research of nanotechnology is one of the important directions for development of modern medicine. In vivo drug delivery is more convenient by nano-particles [14–16]. The in vivo bioavailability of Chinese medicine has been improved, and the clinical efficacy enhanced, which will bring a great breakthrough in Phase I Clinical Trials. In addition, nano-Chinese medicine can be quantified, medicine taking can be more in line with modern medical standards and helpful for its promotion in international market. Furthermore, nanotechnology applied to traditional Chinese medicine manufacturing is expected to prepare modern drugs with the properties of high- and long-lasting efficacy, small doses, low toxicity, easy to take, and good stability.

Nano-liposomes with phospholipid bilayer membrane structure [16]. With excellent hydrophilicity, lipophilicity [17], and the natural targeting, long-term, inclusive and other characteristics of non-toxic, non-immunogenicity, absorption speed, high bioavailability, convenient administration, known as "bio-missile head", can be used as drugs and nutritional factors in the targeting vector [18] and gene technology tool [16–18] that is widely used in medicine, health food, cosmetics, and genetic engineering. Its prominent advantages are smaller than the cells that can be absorbed by tissues and cells with characteristics of sustained release, targeting, protection of drugs, improving the efficacy, and reducing side effects and so on.

Drug absorption and metabolism in a living organism is a complex process. The pharmacological effects of Chinese medicine formulations can not simply be attributed to the chemical compositions, but also to the physical state of the formulations. Therefore, change of the physical state of the pharmaceutical formulations is an effective method for developing new drugs [16].

Experiment

Materials

Escherichia coli (CCTCC AB 91112) was provided by China Center of Type Culture Collection, Wuhan University, People's Republic of China.

Instruments

A microcalorimeter, LKB-2277 Bioassay Monitor manufactured by Thermometric AB, Sweden, was used to obtain the thermogenic curves of the bacteria during growth. With a peristaltic pump and LKB-2219, LKB-2322 device outside the water cycle, each measuring cylinder normally contains a sample and a reference in separate measuring cups (twin system). The heat output from the samples flows from the thermoelectric detector to the large heat sink (in close contact with the water bath). In response, the detector produces a voltage which is proportional to the power output from the sample.

Reagents

Standard artesunate drug was provided by Guilin Pharmaceutical Corp. Ltd. (Guo yao zhun zi H19994073, lot no. 050407); *N*,*N*-dimethylformamide was provided by Shantou City West Longde Chemical.

Methods

Preparation of a peptone culture medium

200 mL of peptone culture medium contained the following: NaCl 1.0 g, peptone 2.0 g, and beef extract 1.2 g. 200 mL of secondary purified water were added and filled into six Erlenmeyer flasks, and then sterilized in high-pressure steam at 120 °C for 35 min. Stored in a refrigerator for usage after cool.

LB liquid culture medium

400 mL of LB culture medium consists of Nacl 2.0 g, yeast 2.0 g, and pancreas peptone 4 g. Twice added purified water to 400 mL and filled into four Erlenmeyer flasks, and then sterilized in high-pressure steam at 120 °C for 35 min. Stored in a refrigerator for usage after cool.

Inoculation of Escherichia coli

1 mL of bacterium from supernatant was drawn using a pipette gun and transferred into the flask with culture medium of *Escherichia coli* on the superpurgative working table. Bacterium was cultured in the thermostat oscillator for 8 h. Took out and stored in a refrigerator for usage.

Sample inoculation

A loopful bacterial culture was inoculated by using an inoculating loop in the test tube with 5 mL LB medium on

the superpurgative working table, respectively, and then sample introduction was performed.

Effects of artesunate on the growth metabolism of Escherichia coli

Artesunate was dissolved in *N*,*N*-dimethylformamide for preparation of 25, 15, 10, 5, and 0.5 mg/mL solution. An accurately measured volume solution (concentration corresponding to 1, 10, 20, 100, and 300 μg/mL) was added in 5 mL *E. coli* LB medium using a pipette gun, respectively. Sample introductions of two different concentrations were performed in sequence twice a day, respectively.

Results

Thermogenic curve of *Escherichia coli* during growth

Using the recorded data, the metabolic thermogenic curve of *Escherichia coli* was measured with an LKB-2277 Bioactivity Monitor (see Fig. 1); The metabolic thermogenic of *E. coli* effected by different concentration artesunate (see Fig. 2); the metabolic thermogenic curves of *E. coli* affected by different concentration artesunate liposomes (see Fig. 3) were plotted as shown in Figs. 1, 2, and 3.

In Figs. 1, 2, and 3, the thermogenic curve of *E. coli* fits well with that of the typical bacterial growth curves obtained by conventional bacteriological techniques during the metabolic process of the lag phase (AB), log phase (BC), stationary phase (CD), and decline phase (DE). The peaks and valleys are the specific features of bacteria in the

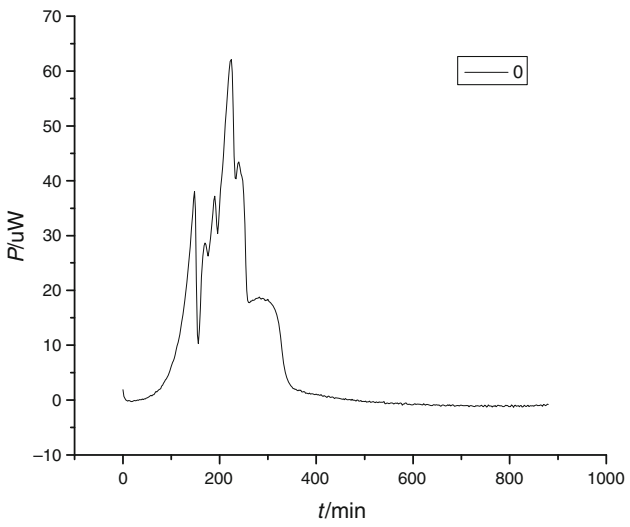

Fig. 1 The metabolic thermogenic curve of *E. coli* was measured with an LKB-2277 Bioactivity Monitor

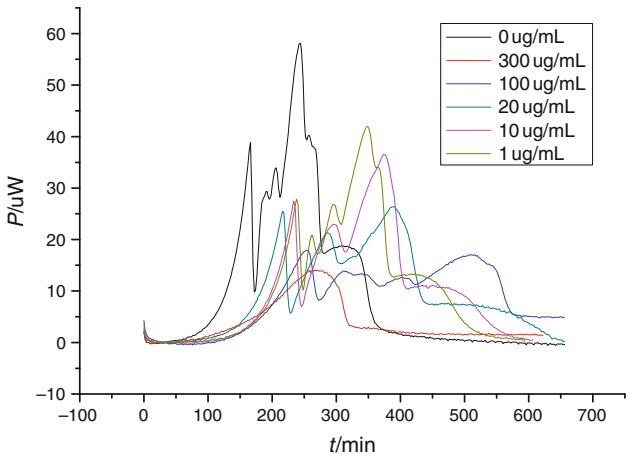

Fig. 2 The metabolic thermogenic curves of *E. coli* affected by different concentration artesunate with were measured with an LKB-2277 Bioactivity Monitor

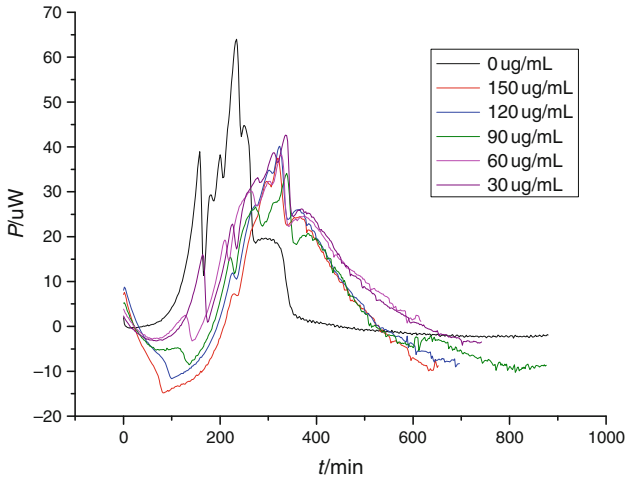

Fig. 3 The metabolic thermogenic curves of *E. coli* affected by different concentrations of artesunate liposomes were determined with an LKB-2277 Bioactivity Monitor

complex media under conditions of limited oxygen. As shown in Figs. 2 and 3, the growth thermogenetic curves of *E. coli* exists remarkable difference in lag phase (AB), log phase (BC), stationary phase (CD), and decline phase (DE).

The inhibitory ratio *I* and the half-inhibitory concentration I_{C50}

The inhibitory ratio *I* and the half-inhibitory concentration I_{C50} at different concentrations of artesunate and artesunate liposomes (see Tables 1 and 2).

Table 1 The inhibitory ratio I and the half-inhibitory concentration I_{C50} at different concentrations of artesunate at 28 °C

C/μg mL^{-1}	k/min^{-1}	R	SD	t_G/min	I/%	I_{C50}/μg mL
0	0.03764	0.9996	0.02754	18.42	0	
1	0.02981	0.99984	0.01481	23.25	20.80	
10	0.03128	0.99934	0.03076	22.16	16.90	137.53
20	0.02964	0.99965	0.01992	23.39	21.25	
100	0.02313	0.99483	0.06818	29.97	38.55	
300	0.01368	0.99713	0.03118	50.71	63.66	

Table 2 The Inhibitory ratio I and the half-inhibitory concentration I_{C50} at different concentrations of artesunate liposomes at 28 °C

C/μg mL^{-1}	k/min^{-1}	R	SD	t_G/min	I/%	I_{C50}/μg mL
0	0.09332	0.99322	0.04571	7.43	0	
30	0.03315	0.99612	0.0272	20.91	64.48	
60	0.04179	0.99326	0.04526	16.59	55.22	50.05
90	0.05441	0.99482	0.03888	12.74	41.7	
120	0.05667	0.99072	0.04554	12.23	39.27	
150	0.07785	0.99047	0.0453	8.9	16.58	

Table 3 Biothermochemical parameters of E. coli affected by different concentrations of artesunate at 28 °C

C/μg mL^{-1}	Total metabolic heat/μW s	Highest point/s	Highest point power/μW
0	5916.67	14040	3722.04
1	369307.50	18000	55880.00
10	361083.80	19080	53385.20
20	332187.00	19920	50559.00
100	253853.50	20880	40554.00
300	43740.00	19200	30526.00

Biothermochemical parameters of *E. coli* affected by artesunate and artesunate liposomes at different concentrations

Biothermochemical parameters of *E. coli* affected by different concentrations of artesunate and artesunate liposomes are shown in Tables 3 and 4.

Linear fit of the data of total metabolic heat of *E. coli* effected by different concentration artesunate (1–300 μg) (see Fig. 4), Eq. 1 was obtained as follows:

$$Y = 364720.61 - 1075.25x,\ R = 0.9985 \quad (1)$$

Fitting the maximum heat production power P_m of *E. coli* against different concentrations of artesunate liposomes (1–700 μg) (see Fig. 5), then

$$Y = 54251.5765 - 35.71122x,\ R = 0.98345 \quad (2)$$

Fitting the data of total metabolic heat output (μW s) of *E. coli* against different concentrations of artesunate (see Fig. 6), then

$$Y = 10121.23 + 310.05507x,\ R = 0.99655 \quad (3)$$

Fitting the data of metabolic heat output (μW s) of *E. coli* against different concentrations of artesunate liposomes (see Fig. 7), then

$$Y = 5001.335 - 41.84305x,\ R = 0.99351 \quad (4)$$

Discussion

Principles

Under non-restrictive conditions, the growth model of bacteria colony obeyed the Malthus equation in the log phase [16]. That is

$$N_t = N_0 \exp(kt) \quad (5)$$

where as K is the rate constant of bacteria growth in the log phase; N_t is the total number of bacteria determined

Table 4 Biothermochemical parameters of *E. coli* affected by different concentrations of artesunate liposomes at 28 °C

C/μg mL^{-1}	Total metabolic heat/μW s	Highest point/s	Highest point power/μW
0	5916.668	14040	3722.035
30	8022.95	18000	4255.7
60	6447.15	19080	3261
90	4541.98	19920	2350.29
120	2250.59	20880	1769.73

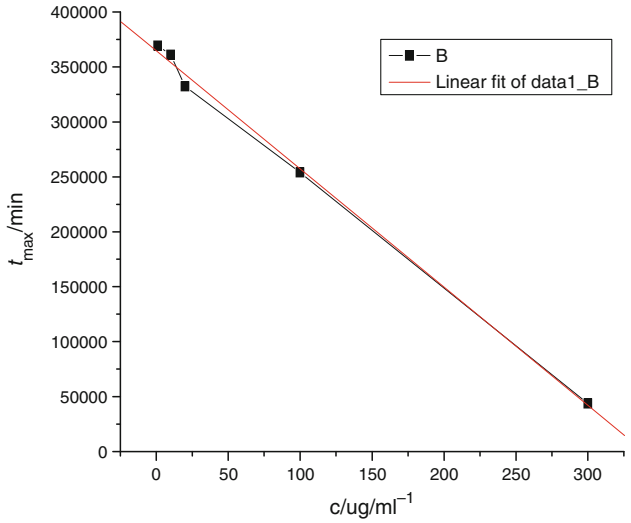

Fig. 4 Total metabolic heat output of *E. coli* affected by different concentration artesunate

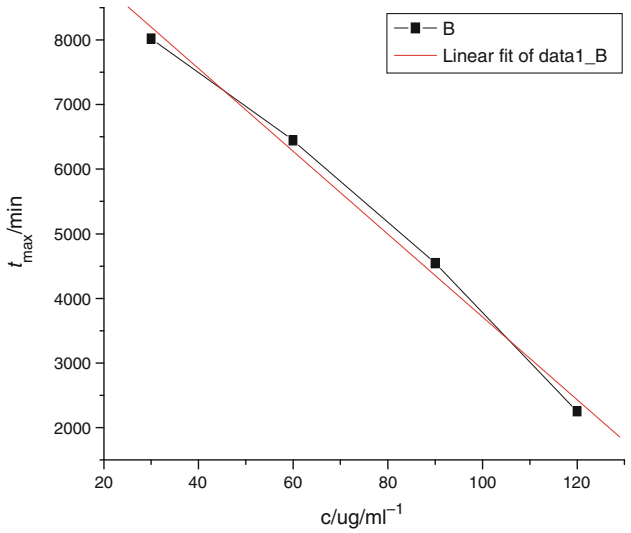

Fig. 5 The highest point power of *E. coli* affected by different concentration artesunate

by real-time; N_0 is the total number of bacteria at time (t_0). Order the bacterial heat output is P, then

$$PN_t = PN_0 \exp(kt) \tag{6}$$

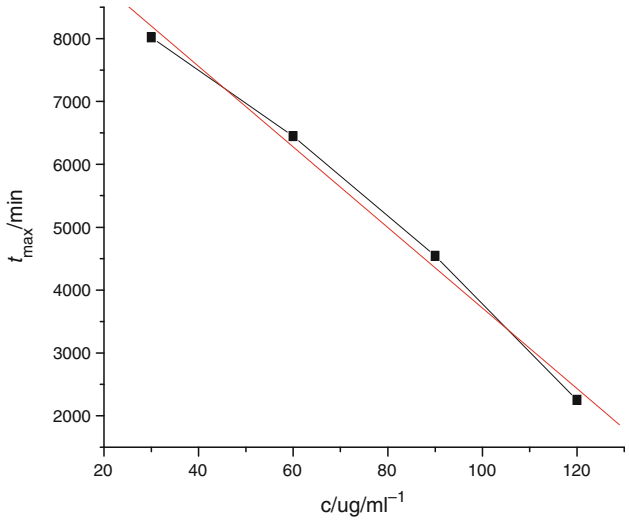

Fig. 6 Total metabolic heat(μW s)of *E. coli* affected by artesunate with different concentrations

where as P is heat output power produced by a single bacterial metabolism. In the log phase of growth metabolism, heat production rate of *E. coli* is growing exponentially [17]. Therefore, the crucial metabolic parameter, the rate constant (k) can be obtained from a kinetic equation:

$$P_t = P_0 \exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \tag{7}$$

where as P_0 is heat production power of the bacterial metabolism at time(t_0) and P_t is heat production power of bacterial metabolism at time t.

Using the data $\ln P_t$ and t taken from the curves to fit Eq. 7, the growth rate constant k of *E. coli* and the corresponding coefficient were obtained. Computer fitting k–c relation, the optimum growth concentration (k at the peak value) and the critical growth concentration C_0 were calculated ($k = 0$). Quantitative analysis of the effects of different drug concentrations on the metabolic activities of *E. coli* was performed. The generation time (G) was derived from the following formula: $t_G = (\ln 2)/k$ [18, 19].

The inhibitory ratio I quantitative analysis of the effects of different concentrations of the drugs on the metabolic activities of the bacteria was defined as:

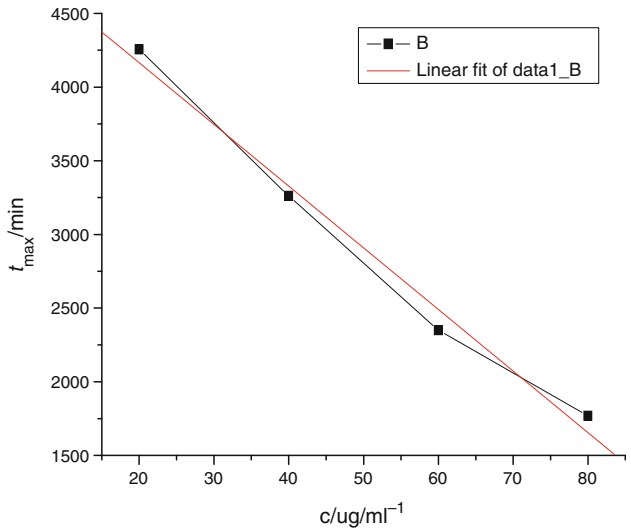

Fig. 7 The highest point power of the total metabolic heat (μW s) of *E. coli* affected by artesunate liposomes at different concentrations

$$I = \frac{k_0 - k_c}{k_0} \times 100\% \quad (8)$$

where as k_0 is the rate constant of the control, k_c is the rate constant of the bacterial growth inhibited by the inhibitor of concentration C. I is the inhibitory ratio of bacterial growth at a certain concentration. The higher the value of I is, the stronger the inhibition of the compound is. When the inhibitory ratio I is 50%, the corresponding concentration of inhibitor is called the half-inhibitory concentration I_{C50}. Using the half-inhibitory concentration I_{C50}, quantitative comparison of the effects of different compounds on the metabolic activities of the same bacteria can be made, and a screening model of the complexes based on "inhibition of bacterial growth" is established [20].

The results show that (1) affected by artesunate liposomes and artesunate, respectively, the thermogenic curves of *E. coli* are obviously different in the metabolic process of the lag phase (AB), log phase (BC), stationary phase (CD), and decline phase (DE); (2) artesunate liposomes drive an endothermic reaction taking place in the bacterial solution; (3) inhibition of artesunate liposomes is much stronger than that of artesunate; (4) compare with artesunate, artesunate liposomes possessed a sustained release property; (5) a comparison of the inhibitory ratio I and the half-inhibitory concentration I_{C50}, *E. coli* exhibits a difference in sensitivity between artesunate liposomes and artesunate; (6) further study is still needed for better understanding of the endothermic reaction created by artesunate liposomes and the process of the approach [21].

A contrast of the inhibitory ratio I and the half-inhibitory concentration I_{C50}, we found that *E. coli* exhibits a difference in sensitivity between artesunate liposomes and artesunate.

Artesunate liposomes drive an endothermic reaction taking place in the bacterial solution; the real mechanism needs further research.

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References

1. Wang H, Wang J-Y. Research progress in antimalarial mechanism and drug-resistant mechanism of artemisinin and its derivative. Int Phar J. 2007;34:36–8.
2. Dhingra V, Rao KV, Narasu ML. Current status of artemisinin and its derivatives as antimalarial drugs. Life Sci. 1999;66:279–300. doi:10.1016/S0024-3205(99)00356-2.
3. Krishna S, Bustamante L, Haynes RK, Staines HM. Artemisinins: their growing importance in medicine. Trends Pharmacol Sci. 2008;29:520–7.
4. Sen R, Ganguly S. Efficacy of artemisinin in experimental visceral leishmaniasis. J Antimicrob Agents. 2010;36:43–9.
5. Zheng D, Liu Y, Zhang Y, Chen XJ, Shen YF. Microcalorimetric investigation of the toxic action of Cr(VI) on the metabolism of *Tetrahymena thermophila* BF5 during growth. Environ Toxicol Pharmacol. 2006;22:121–7.
6. Oh S, Jeonga IH, Shinb W-S, et al. Growth inhibition activity of thioacetal artemisinin derivatives against human umbilical vein endothelial cells. Bioorg Med Chem Lett. 2003;13:3665–8. doi:10.1016/j.bmcl.2003.08.023.
7. Holzel R, Motzkus C, Lamprechet I. Kinetic investigation of microbial metabolism. Thermochim Acta. 1994;239:17–24.
8. Vig Andrea, Dudás Réka, Kupi Tünde, Orbán J, Hild G, Lőrinczy D, Nyitrai M. Effect of phalloidin on filaments polymerized from heart muscle adp-actin monomers. J Therm Anal Calorim. 2009;95:721–5. doi:10.1007/s10973-008-9404-5.
9. Vogt RL, Dippold L. *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef. Public Health Rep. 2005;120:174–8.
10. Ohman L, Normann B, Stendahl O. Physicochemical surface properties of *Escherichia coli* strains isolated from different types of urinary tract infections. Infect Immun. 1981;32:951–5.
11. Aarestrup FM. Monitoring of antimicrobial resistance among food animals: principles and limitations. J Vet Med B. 2004;51:380–8.
12. Fish JT, Pettibone GW. Influence of freshwater sediment on the survival of *Escherichia coli* wand *Salmonella* sp. as measured by three methods of enumeration. Lett Appl Microbiol. 1995;20:277–81.
13. Schraft H, Watterworth LA. Enumeration of heterotrophs, fecal coliforms and *Escherichia coli* in water: comparison of 3 M^{TM} Petrifilm^{TM} plates with standard plating procedures. J Microbiol Method. 2005;60:335–42.
14. Villaverde A. Nanotechnology, bionanotechnology and microbial cell factories. Microb Cell Fact. 2010;9:53. doi:10.1186/1475-2859-9-53.
15. Curtis AS, Dalby M, Gadegaard N. Cell signaling arising from nanotopography: implications for nanomedical devices. Nanomed. 2006;1:67–72. doi:10.2217/17435889.1.1.67.
16. Carmona-Ribeiro AM. Bilayer-forming synthetic lipids: drugs or carriers? Curr Med Chem. 2003;10:2425–46.
17. Skar-Gislinge N, Simonsen JB, Mortensen K, et al. Elliptical structure of phospholipid bilayer nanodiscs encapsulated by

scaffold proteins: casting the roles of the lipids and the protein. J Am Chem Soc. 2010;132:13713–22. doi:10.1021/ja1030613.
18. O'Hagan DT, Singh M, Ulmer JB. Microparticles for the delivery of DNA vaccines. Immunol Rev. 2004;199:191–200.
19. Levy G, Matsuzawa T. Effect of complex formation on drug absorption II. Lipoid-soluble dye complexes. J Pharm Sci. 2006; 54:1003–6. doi:10.1002/jps.2600540710.
20. Li XC, Hong W, Sheng QS. Microcalorimetric study on the aerobic growth of *Escherichia coli*. Thermochimica Acta. 1995; 253:175–82.
21. Zhu J-C, Liu Y, Wong W-K. Investigation of antibacterial activity of two kinds of novel Schiff Bases on *Escherichia coli* by microcalorimetry. Chinese J Chem. 2006;24:1295–300.