Journal of Food Engineering 131 (2014) 26–32

Contents lists available at ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

Effects of pulsed electric fields on the permeabilization of calcein-filled soybean lecithin vesicles

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ARTICLE INFO

Article history:
Received 22 October 2013
Received in revised form 15 December 2013
Accepted 12 January 2014
Available online 22 January 2014

Keywords:
Pulsed electric fields
PEF
Cell membrane
Permeabilization
Calcein
Soybean lecithin vesicles

ABSTRACT

Effects of PEF on permeabilization of calcein-filled large unilamellar vesicles (LUV) with average diameter of 1705.64 ± 55.91 nm and small unilamellar vesicles (SUV) with average diameter of 213.06 ± 5.50 nm, suspended in an isosmotic sucrose solution (150 mM) were investigated. The suspensions of vesicles were exposed to PEF at various electric field strengths in the interval of 0–50 kV/cm and different treatment times ranging from 0 to 4.8 ms. The intensity of permeabilization induced by PEF was analyzed in terms of the changes of fluorescence intensity of the suspension solution and the average diameter of vesicles, due to the leakage of self-quench calcein encapsulated in vesicles. Results indicated that the permeability of LUV was increased with increasing intensity of electric field (>10 kV/cm) and treatment time, and the release percentage of calcein (*R* value) was increased by 57.98% (from 0.46% to 58.44%) with the increase of the electric field strength (from 10 to 50 kV/cm) at 4.8 ms. However, the R value was only increased by 14.62% (from 43.82% to 58.44%) with the increase of the treatment time (from 1.2 to 4.8 ms) at 50 kV/cm. Average diameter of LUV was sharply decreased from 1705.64 ± 55.91 to 465.60 ± 37.12 nm with the maximum value at 50 kV/cm and 4.8 ms. Results also showed that shrinkage or rupture of LUV happened, as confirmed by the change of size distribution of LUV after treatment by PEF. However, as for SUV, the changes of the permeability of vesicles and average diameter were not observed. In addition, it was demonstrated that the magnitude of the induced transmembrane potential was proportional to the radius of the vesicle, and larger vesicles could be polarized or ruptured at relatively lower field strength than smaller vesicles.

1. Introduction

As one of the novel food processing technologies (Delgado et al., 2009; Du and Sun, 2005; Jackman et al., 2008; Li and Sun, 2002; Sun and Li, 2003; Sun and Zheng, 2006; Valous et al., 2009; Wang and Sun, 2001; Zheng and Sun, 2006), pulsed electric field (PEF) is widely used to inactivate food-borne microorganisms and endogenous enzymes in liquid foods (Liu et al., 1997; Reina et al., 1998; Saldana et al., 2010; Zhao et al., 2012a,b), extract intracellular bioactive compounds from plant tissue and yeasts (Corrales et al., 2008; López et al., 2009; Liu et al., 2011a,b), modify polymer macromolecules (Liu et al., 2011a,b; Luo et al., 2010), promote non-catalysis chemical reactions (Guan et al., 2010; Lin et al., 2012), and so on. With little altering of nutritional and sensorial food properties and with low energy consumption due to its non-thermal nature, PEF processing technology has received great attention in recent years (Abenoza et al., 2013; Li et al., 2013; Marsellés-Fontanet et al., 2013; Monfort et al., 2013; Parniakov et al., 2014; Zhang et al., 2013; Zhao et al., 2014), which provides potential alternatives to traditional thermal treatment techniques.

For microbial inactivation, high electric field ranging from 10 to 50 kV/cm with short duration from 1 to 100 μs is applied to liquid food flowing between two electrodes, and the underlying mechanisms and intracellular substance extraction by PEF are attributed to the permeabilization change and loss of cell membrane integrity when the applied electric field exceeds a critical value (Barsotti and Cheftel, 1999). The interaction of electric fields with lipid membrane and cells has been extensively studied in the last decades (Mir et al., 1997). Under normal physiological conditions, cell membrane is impermeable to ions and hydrophilic molecules. When cell membrane is exposed to sufficiently high external electric fields, it may result in the generation of pores. Thus the membrane becomes transient permeable. Depending on the extent of applied parameters, such as electric field strength, treatment

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http://dx.doi.org/10.1016/j.jfoodeng.2014.01.009
0260-8774/

time and pulse duration, this process can be irreversible or reversible (Aronsson et al., 2005; Zhao et al., 2012a,b). Several studies about permeabilization induced by electric field have already been conducted in an attempt to optimize and model the influence of electric field parameters, including intensity and duration (Sens and Isambert, 2002; Shillcock and Lipowsky, 2005; Staykova et al., 2008). Although many studies on the phenomenology of cell electropolarization and cell membrane electroporation have already been reported (Stromberg et al., 2000; Teissie and Tsong, 1981; Tekle et al., 2001; Veatch and Keller, 2002), the mechanism of pore opening across the lipid membrane is still not fully understood because the electroporation of cell membrane is a very short timescale (ns) process and it is difficult to be observed directly.

Factors that affect the efficiency of inactivation microorganism in liquid foods can be divided into three types: (1) PEF parameters, such as electric field strength, pulse duration and pulse numbers; (2) microorganism characteristics, such as growth condition, growth phase, recovery condition and sizes of microorganisms; and (3) environmental conditions, such as composition of products, ionic strength, conductivity and pH (Saulis, 2010; Wouters et al., 2001). Among these factors, the impact of the size of microorganisms on inactivation efficiency may be much more significant. Due to the heterogeneousness of different cells, the threshold value of the electric field strength required to generate transmembrane potential is discrepant. For example, as plant tissue cells are several hundred micrometers in size, which are much bigger than microorganisms ranging from 0.1 to 15 μm, vegetable cells are much more sensitive to PEF treatment than yeast, bacteria and virus (Dymek et al., 2013; Saldana et al., 2010). Previous studies also reported that small microorganism cells were found to be less sensitive against PEF. For example, it was reported that *Listeria monocytogenes* with sizes ranging from 0.4 to 2 μm were much more resistant to PEF treatment than other bigger bacterium species such as *Escherichia coli* and *Salmonella senftenberg* (Saldana et al., 2010). In addition, Khadre and Yousef (2002) reported that it was hard to use PEF treatment to sterilize rotavirus with sizes ranging from 0.1 to 0.4 μm even at high intensity due to the small sizes of treated subjects. Furthermore, Golberg et al. (2012) reported that *Listeria monocytogenes* modified genetically with different cell membrane had different electroporation thresholds. Despite the above studies, information on the permeabilization of cell membrane as affected by sizes of microorganism treated by PEF is still limited.

Therefore, the aim of this research was to explore the electroporation behavior of vesicles with different sizes under PEF by means of measuring the release of fluorescence probe substance – calcein. Soybean lecithin vesicles with different diameters were used to simulate the cell membrane and exclude the interference of other substances in a living cell. The correlations between average diameter and the ratio of released calcein of the intact and damaged vesicles were also established in the current study.

2. Materials and methods

2.1. Materials

Soybean lecithin (phosphatidylcholine >90%) and calcein were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China), Chloroform and diethyl ether were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were of reagent grade.

2.2. Liposome preparation

Large unilamellar vesicles (LUV) were prepared from soybean lecithin by the reverse evaporation method (Szoka and Papahadjopoulos, 1978), with slight modification. An amount of 250 mg lecithin was dissolved into chloroform in a 50-mL round-bottomed flask. The organic solvent was evaporated by a rotary evaporator (Yalong Instrument Co. Ltd., Shanghai, China) under vacuum, leaving a uniform thin film on the wall of the flask. Diethyl ether was then used to dissolve the thin film and 50 mM calcein solution with amount ratio of 1/3 to diethyl ether by volume was added. Thereafter, the mixture of diethyl ether and calcein solution was sonicated (2–5 min) in a bath-type sonicator (Ningbo Xinzi Biotechnology Instrument Co. Ltd., Ningbo, China) with power of 500 W and temperature under 10 °C. The mixture was then placed on the rotary evaporator and the organic solvent was removed under vacuum at 0–10 °C. Then 10 mM Tris–HCl and 70 mM NaCl buffer solution were added to re-suspend liposomes and the suspension was evaporated for an additional 15 min at 20 °C to remove traces of solvent. Five freeze–thawed cycles were conducted by a lyophilizer (SCIENIZ-18N, Ningbo Xinzi Biotechnology Instrument Co. Ltd., Ningbo, China) to obtain solute equilibrium between the trapped and bulk solutions. Unincorporated calcein was removed from the liposomes by repeated centrifugal washes in buffer using a supercentrifuge (14,000×*g*) (JW-3021HR, Anhui Jiaven Equipment Industry Co. Ltd., Anqing, China). Finally, the vesicle suspension was diluted with isotonic degassed sucrose solution (150 mM sucrose) to prepare aliquots with a final lipid concentration of about 0.30 mg/mL. The electrical conductivity of the suspension was 15.60 μS/cm measured by a conductivity meter (DDS-11A, Shanghai Leici-Chuangyi Instrument and Meter Co. Ltd., Shanghai, China) at 25 °C. The average diameter of LUV was 1705.64 ± 55.91 nm as measured by a particle size distribution analyzer (LB-550, Horiba Ltd., Tokyo, Japan).

Small unilamellar vesicles (SUV) were prepared based on the similar procedure described above with slight modification: after freeze–thawed five times by the lyophilizer, the vesicle suspension flew through a polycarbonate membrane (200 nm pore size, Millipore®, Millipore Ltd., Billerica, USA) at room temperature of 25 °C for 15 times. To remove the unencapsulated calcein, the vesicles suspension was eluted through a Sephadex G-50 gel permeation column (Amersham Pharmacia Biotech, Piscataway, New Jersey). The average diameter of LUV was 213.06 ± 5.50 nm as measured by the particle size distribution analyzer.

2.3. PEF processing system

The PEF treatment was performed in a continuous PEF system (SCUT PEF Team, Guangzhou, China), described in previous studies (Han et al., 2012; Liu et al., 2011a,b). Fig. 1 shows the PEF treatment for the vesicles solution.. The following PEF treatment conditions were used: unipolar square wave, pulse frequency of 1 kHz, pulse duration of 40 μs, electrode distance of 0.30 cm, flow volume of 0.02 mL in the treatment chamber, and sample flow rate of 0.50 mL/s. The applied electric field intensity was 0, 10, 20, 30, 40, 50 kV/cm, respectively. The PEF treatment time t (s) was calculated with the number of pulses N_p received in the chamber, and the pulse duration W_p (μs) as shown below:

$$t = N_p \times W_p \tag{1}$$

where N_p can be determined as:

$$N_p = (V/F) \times f \tag{2}$$

where f is the pulse repetition rate, V (mL) is the volume of the chamber and F (mL/s) is the flow rate.

In the current experiment, as calculated by Eq. (1) and (2), the treatment time for individual treatment was 1.2 ms. If the sample needs to be treated for 4.8 ms, the treatment should be circulated for 4 times. For comparison, experiments with control samples were performed using the same procedure without PEF treatment