

Influence of Natural Inorganic Impurity on the Synthesis of Poly(L-lactic acid), Properties and Nanofabrication

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ABSTRACT: This work is focused on determination of the effect of a calcium-based inorganic impurity originating from biotechnological L-lactic acid production from cheese whey on the synthesis of poly(L-lactic acid). The influence of the impurity was investigated in the range of 0 to 1 wt % and the product properties were studied by analytical techniques GPC, FTIR-ATR, acid end group analysis and DSC. The final product was used to prepare submicron particles, which were analyzed by dynamic light scattering and electron microscopy. Results show a moderate reduction in molecular weight in connection with an increase in the amount of the impurity up to 0.75 wt %. Further increase in the concentration of the impurity caused decomposition. Investigating the thermal properties reveals a noticeable reduction in the melting temperature and a slight decrease in glass transition temperature. The prepared submicron particles had a Z-average diameter of between 280 and 520 nm, depending on the emulsifying system, and the presence of the inorganic impurity seems to play no role during particle formation. © 2013 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* 130: 63–69, 2013

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INTRODUCTION

Biodegradable polymers have attracted much attention in recent decades due to their unique properties such as bioresorbability, biodegradability, the availability of renewable resources and their great potential to replace synthetic polymers.^{1–3} In particular, polymers from renewable and sustainable resources are in the spotlight due to their “green” qualities.

Poly(L-lactic acid) (PLLA) is one of the most promising biodegradable polymer, which can be obtained from inexpensive renewable resources, e.g., polysaccharides. PLLA belongs to a varied group of polyesters and can be applied in packaging technology as well as in the medical field, e.g., in materials for orthopedics, sutures, nanoscaffolds, and drug delivery, due to its biocompatibility and bioresorbability.³

The monomer for PLLA synthesis, 2-L-hydroxypropanoic acid, better known under the trivial name L-lactic acid (LA), can be produced either through synthetics or a biotechnological pathway. The latter method usually includes microbial fermentation

of simple saccharidic substrate into LA followed by purification as shown in Figure 1.

First, the fermentation medium that consists of saccharides, bacteria and supplement nutrients is mixed together and fermented at a temperature of ~42°C into LA. Second, in order to raise the pH during acid production, Ca(OH)₂ (or another base) is continuously added. This leads to the conversion of LA into its salt—calcium lactate, which must be converted back into acid form; for example, via a reaction with H₂SO₄. Although fermentation and conversion usually produce a good yield, the product still contains residual impurities—e.g., citric acid, acetic acid, residual lactose, protein and inorganic compounds—which must be removed. However, the purification of crude LA is known to be the most expensive part of whole process.⁴

One of the promising resources for lactic acid production can be a cheese whey; a by-product of the dairy industry. Its production in high volume and at low cost makes it more than suitable as a base material for fermentation.⁶ Because of the fact

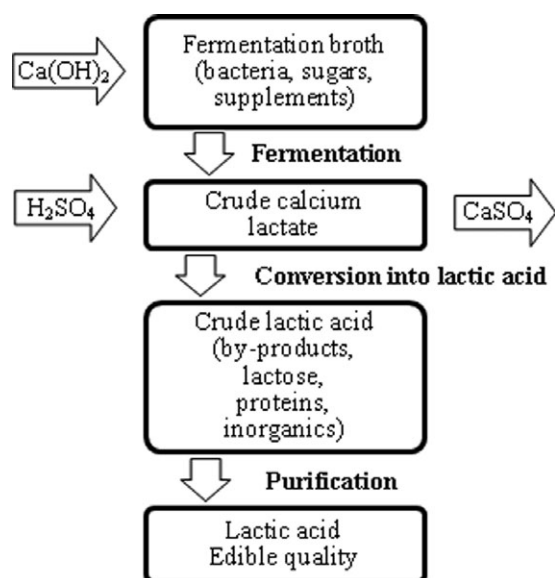


Figure 1. Traditional lactic acid production process.⁵

that the whey is often considered undesirable waste, there is a significant interest in finding a new method for its utilization.^{6,7}

The polymerization step required LA of high monomer purity, which is technologically and financially demanding. However, as was previously mentioned, biotechnologically prepared LA can contain impurities such as organic acids, residual proteins, and inorganic compounds, which could affect the polymerization step.

Purifying LA represents an additional economic load, which reduces the competitiveness of this polymer in comparison with petroleum-based plastics. In the authors' previous works research focused on the influence of citric acid as a by-product of fermentation and a proteinic impurity on the polycondensates of LA. It was found that the presence of citric acid reduces the quality of the resulting polymer significantly (in terms of molecular weight). A slight deterioration in thermal properties was observed due to a residual protein impurity in LA.^{8,9}

The aim of this particular work is to determine the effects of a residual calcium inorganic impurity in LA as a monomer for PLLA production through a direct polycondensation reaction catalyzed by tin II-ethylhexanoate. Milk calcium (hereinafter referred as Lactoval) was used as the calcium inorganic residuum. The results were quantified by observing the changes in molecular weight distribution, physicochemical composition and thermal properties. The suitability of the final product as a potential material for producing submicron particles was also examined.

EXPERIMENTAL

Materials

L-lactic acid (LA) of 80% water solution, optical purity 99% (measured on an Optech P1000 polarimeter at 22°C, with a concentration of 10%) was purchased from Lachner Neratovice, the Czech Republic. Lactoval HiCal (the properties of which are summarized in Table I) was supplied by DMW International.

Table I. Detailed Composition of Lactoval

Parameter	Amount
Calcium (70% phosphate, 20% lactate, 10% citrate)	27.9%
Proteins	2.5
Moisture	5.4
pH (10% water, 20°C)	7.1
Scorched particles	ADPI A
Standard plate count	500 cfu/g
Enterobacteriaceae	<1 cfu/g
Yeasts and moulds	10 cfu/g
CP staphylococcus in 1g	negative
Sulphite reducing Clostridia	10 cfu/g
Salmonella in 50 g	negative

The following—stannous 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$, 95%), sorbitan monopalmitate (Span40), polyoxyethylene-polyoxypropylene block copolymer (Pluronic F68), polyethylene glycol sorbitan monolaurate (Tween20), gelatin type A (Gelatin A)—were supplied by Sigma Aldrich, Steinheim, Germany. The solvents acetone, chloroform and methanol (all of analytical grade) were bought from IPL Lukes, Uhersky Brod, the Czech Republic.

Sample Preparation

A predetermined amount of LA and Lactoval (Table II) was added to a double-necked flask (250 mL) equipped with a Teflon stirrer. The flask was then placed in an oil bath heated by a magnetic stirrer with heating and connected to laboratory apparatus for distillation under reduced pressure. The dehydration step followed (in all cases; at 160°C, 15 kPa, 4 h, continuous mixing at 250 rpm). After LA dehydration, the reactor was disconnected from the vacuum pump, and the relevant amount of 0.2 mL of $\text{Sn}(\text{Oct})_2$ was added. Then the reaction continued for 24 h at 160°C and a pressure of 100 Pa. The resultant product, in the form of a colorless or light brown viscous melt, was allowed to cool down to room temperature and dissolved in acetone. Then the polymer solution was precipitated in a mixture of chilled methanol/distilled water at the ratio 1:1. The powder obtained was filtered, washed with methanol, and dried under a reduced pressure of 20 kPa at 45°C for 48 h.

Submicron particles were prepared by the solvent evaporation technique according to a method described in the authors' previous work.¹⁰ In brief, the polymer was dissolved in chloroform (400 mg of polymer in 4 mL of chloroform). Second, the polymer solution was emulsified in a water solution of various emulsifying systems under continuous stirring with the homogenizer DI 18 basic (Yellow line by IKA, Belgium) at a stirring speed of 24 000 rpm. The water: chloroform ratio was 4:1 in all experiments. The obtained emulsion was ultrasonically dispersed by an ultrasonic probe (Hielscher UP 400S, Germany) for 5 min (cycle 0.5; amplitude 35%). Finally, the organic solvent was evaporated from the emulsion under a reduced pressure of 20 kPa.

Table II. Summary of Experiment Composition and Main GPC and End Group Analysis Results

Lactoval (wt %)	Calcium ^a (wt %)	Protein ^a (wt %)	Lactic acid in flask (mL)	Yield ^b (%)	[η] (dL/g)	M_n^c (g/mol)	\mathcal{D}_M^c	AN (g_{KOH}/g_{sample})
0.00	0.00	0.000	52.1	61.7	0.47	22600	1.50	24.8
0.18	0.05	0.004	51.99	51.1	0.25	26900	1.31	30.7
0.36	0.10	0.009	51.90	54.6	0.24	24400	1.47	36.2
0.50	0.14	0.010	51.82	55.5	0.24	24780	1.32	39.2
0.73	0.20	0.018	51.71	56.4	0.24	20000	1.56	37.5
1.00	0.28	0.025	51.56	0.0	-	-	-	-

^aCalculated from Lactoval composition given in Table I, ^bCalculated to initial amount of LA, ^cObtained from GPC analysis.

Sample Characterization

GPC analysis was conducted using the Agilent PL GPC 220 chromatographic system. Samples were prepared by adding 1 mL of chloroform to ~ 1.5 mg of polymer. Separation and detection took place on a PL gel-mixed-D bed column (300×7.8 mm², 5 μ m particles) with a RI response detector. Analyses were carried out at 30°C with a chloroform flow rate of 1.0 mL min⁻¹ and a 100 μ L injection loop. The GPC system was calibrated with narrow polystyrene standards ranging from 580 g mol⁻¹ to 271,000 g mol⁻¹ (Polymer Laboratories, the United Kingdom). The weight average molar mass M_w , number average molar mass M_n and molar-mass dispersity ($\mathcal{D}_M = M_w/M_n$) of the products were determined from their peaks corresponding to polymer fraction, and expressed as 'polystyrene equivalent' molecular weights.

Viscosity measurements were performed in CHCl₃ at 30°C on an Ubbelohde viscometer with capillary type 0a (inner diameter 0.46 mm). Intrinsic viscosity ($[\eta]$) was calculated from eq. (1):

$$[\eta] \text{ (dL g}^{-1}\text{)} = \lim_{c \rightarrow 0} \left(\frac{\eta_{rel} - 1}{c} \right) \quad (1)$$

where η_{rel} is the relative viscosity, which is equal to the ratio of the polymer solution and pure solvent viscosities, whereas c is the concentration of the polymer solution (0.4, 0.8, and 1.2 wt/vol %).

The concentration of terminal carboxyl groups was expressed as an acidity number (AN), which represents the amount of KOH (in milligrams) necessary for neutralizing 1 g of a sample. AN was determined by titration when ~ 0.2 g of the product was dissolved in dichloromethane and titrated with 0.01M KOH solution (in ethanol). The bromothymol was used blue as an indicator and calculation was performed according to eq. (2).

$$AN \text{ (mg g}^{-1}\text{)} = \frac{V \times c \times 56.1}{w} \quad (2)$$

where V is the volume of the KOH solution required for titration, c is the concentration of the KOH solution and w is the weight of a sample.

The physicochemical composition of the product was identified using Fourier transform infrared analysis (FTIR). The investiga-

tion was conducted on the NICOLET 320 FTIR, equipped with an attenuated total reflectance (ATR) accessory utilizing Zn-Se crystal and the software package OMNIC. The spectra were collected over the range of 400–4000 cm⁻¹ and resolution of 2 cm⁻¹.

Differential scanning calorimetry (DSC) was used to analyze the thermal properties of the polycondensation products. The experiments were performed on the Mettler Toledo DSC1 STAR system under a nitrogen atmosphere (20 cm³ min⁻¹) according to the following program: (i) first heating scan 0°C–170°C (20°C min⁻¹); (ii) annealing at 170°C for 1 min; (iii) cooling scan 170°C–0°C (20°C min⁻¹); (iv) annealing at 0°C for 1 min; (v) second heating scan 0°C–95°C (20°C min⁻¹). The melting point temperature (T_m) as well as the exothermal response relating to crystallization temperature (T_c) was obtained from the first heating cycle. The value of glass-transition temperature (T_g) was determined in the second heating scan at the midpoint stepwise increase of the specific heat associated with glass transition.

Evaluation of particle size distribution (PSD) and Z-average diameter (ZD) was performed on a Zetasizer (Zetasizer Nano ZS, Malvern Instruments) following the manufacturer's instructions. The morphology of particles was investigated by scanning electron microscopy (SEM; VEGA-II, Tescan, the Czech Republic). Particles were washed with distilled water three times to remove soluble substances from their surface, dried at laboratory temperature and coated with a gold–platinum layer. The electron accelerating voltage was 10 kV.

RESULTS AND DISCUSSION

Figure 2 shows the dependence of M_w of the products in line with the increasing Lactoval content. It can be seen that even a trace of the inorganic impurity (0.18 wt %) led to a significant drop in M_w (from 47,000 g mol⁻¹ to 35,000 g mol⁻¹), which was nearly 75% of the initial value. However, this trend did not continue with further concentrated accrual of Lactoval in the reaction mixture, and only a moderate M_w decrease (to 31,200 g mol⁻¹) was observed. Additionally increasing the Lactoval concentration resulted in total decomposition of the mixture and all of the product was converted to white solid residuum in the condenser, composed of a mixture of lactide, LA and

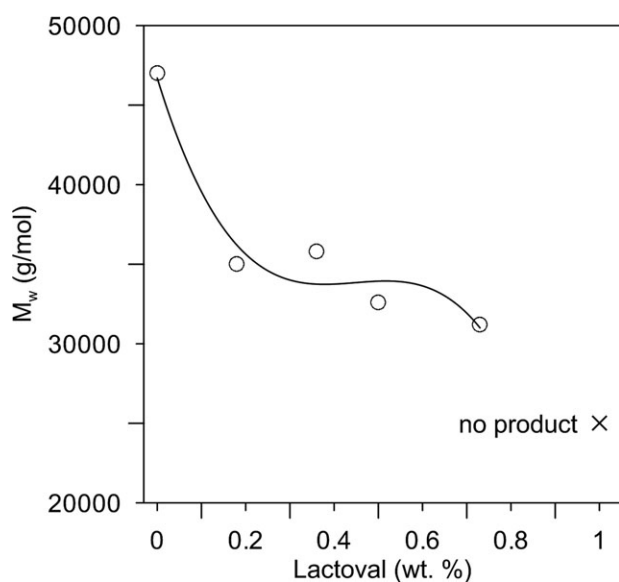


Figure 2. Dependence of M_w on inorganic impurity concentration.

oligomers extensively formed by sublimation of the product under high temperature and vacuum conditions.¹¹

Figure 3 shows neat chromatograms such as they were obtained from the UV detector at 254 nm. It can be clearly discerned that, excluding the polymer peak, low molecular weight compounds were also present in the sample. These fractions are probably attributable to oligomers with short chain lengths, unreacted monomer or other impurities. An M_w of the products of $\sim 35,000 \text{ g mol}^{-1}$ is sufficient for some applications as a drug

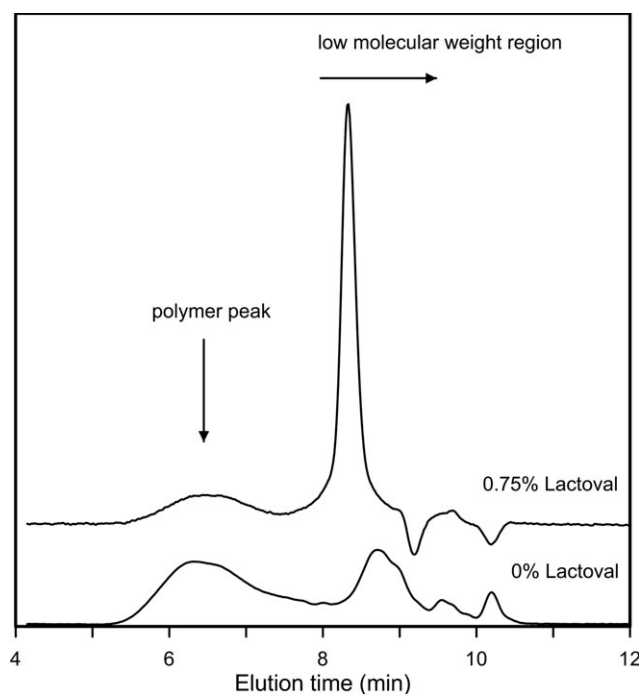


Figure 3. Chromatograms of prepared polymers.

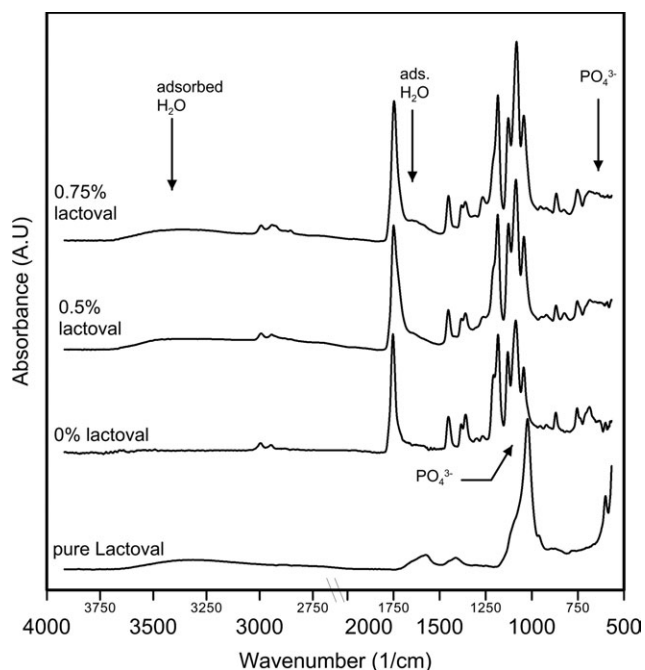


Figure 4. FTIR-ATR spectra of Lactoval, neat PLA and products containing Lactoval.

delivery matrix, for microencapsulation or further copolymerization.

The same trend as exhibited by M_w was observed for intrinsic viscosity (Table II), which is directly proportional to molecular weight. It may be stated that the calcium-based inorganic impurity had a negative effect on the resulting molecular weights; however, this effect was relatively poor in contrast with, for example, the acidic impurity discussed in the authors' previous paper and comparable with the presence of a pure protein impurity.^{8,9}

FTIR-ATR analysis of neat Lactoval and the products is presented in Figure 4. It can be seen that the spectrum of Lactoval contains several typical absorption peaks related to phosphate compounds. The strong signals at 600 cm^{-1} and 1100 cm^{-1} are typical for PO_4^{3-} , while the broad peak between 3000 and 3500 cm^{-1} and the peak at 1650 cm^{-1} were associated to molecularly adsorbed water.¹² The spectrum of pure PLA (without any impurity) showed typical absorption regions for this material. Its detailed description can be found in many sources of the literature.^{3,13} Examples of the main signals are: 2997 cm^{-1} and 2945 cm^{-1} ($-\text{CH}-$ stretching), 1750 cm^{-1} ($-\text{C}=\text{O}$) absorbance region, 1300 – 1050 cm^{-1} ($\text{C}-\text{O}-\text{C}$ stretching), and 1046 cm^{-1} ($\text{C}-\text{CH}_3$ stretching). The signals at 600 cm^{-1} , 3300 cm^{-1} , and 1650 cm^{-1} , which are missing from the spectrum of pure PLA, show upon the presence of residual phosphate compounds in products containing Lactoval. The absence of the strongest PO_4^{3-} signal at 1100 cm^{-1} in the final products was probably caused by its overlapping with ($\text{C}-\text{O}-\text{C}$ stretching) peaks.

Comparing the AN, it can be seen that this generally increased in accordance with rising Lactoval concentration. This is a consequence of the dropping molecular weight when unreacted

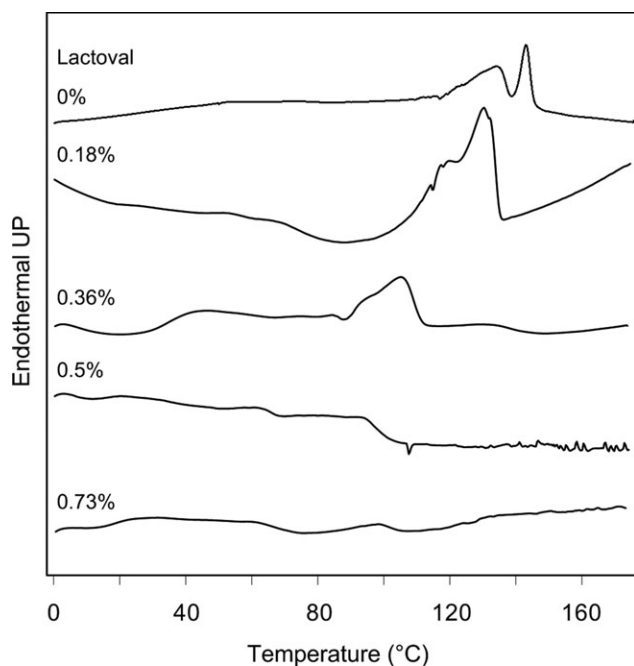


Figure 5. First DSC scans of products containing various levels of impurity concentration.

COOH groups increased in AN. The most crucial information from this experiment relates to the fact that although M_w fell in line with rising Lactoval concentration, the COOH groups remained reactive, hence available for possible further reaction. It can be concluded that Lactoval did not directly react with LA, as this would have meant the reaction would stop and merely a very low molecular weight product would be achieved, as was observed in previous works by the authors on citric acid.⁸

The effect of the presence of the inorganic impurity on thermal behavior is shown in Figure 5 and the data are summarized in Table III. It can be discerned that pure PLLA exhibited two distinctly separated endothermal peaks related to the melting regions of the PLLA crystals.

The absence of a cold crystallization peak and not clearly resolved glass transition region ($\sim 40^\circ\text{C}$) was demonstrated on the crystalline nature of the samples. With a Lactoval concentration of 0.18 wt %, both T_m s were depressed (by $\sim 13^\circ\text{C}$) and a cold crystallization peak appeared at 86.5°C . The two melting

peaks could indicate the presence of different types of crystals with different stabilities or the presence of less perfect crystals which had enough time to melt and rearranged into crystals with better structural perfection, which remelted at higher temperature.^{14–17} At Lactoval concentration 0.36% the cold crystallization peak was not reliably detected, which could be caused by too fast heating rate.

The trend of T_m depression continued in relation to the increasing concentration of the impurity, and at 0.5 wt % the melting endotherm became almost undetectable. This led to the conclusion that the inorganic calcium impurity negatively influenced the crystalline phase of PLLA and restricted the mobility of chains.

Monitoring the T_g (data summarized in Table II) shows a similar course as was observed for T_m s. The presence of the inorganic impurity led to a decrease in T_g between 2°C and 5°C with no clear composition dependence. This decrease might be attributed to a drop in M_w and the increasing number of end groups, T_g . The latter is supported by the acidity number, which increased in accordance with a rise in the concentration of Lactoval.

Figure 6 shows the Z-average diameter of the prepared submicron particles, which were formed from a product containing 0.5% with a varied emulsifying system. It is apparent that all the systems exhibited a Z-average diameter of below $1\ \mu\text{m}$, while the highest value ($\sim 520\ \text{nm}$) was achieved with the Gelatin A/Pluronic F68 system. Nevertheless, the remainder of the emulsifying systems provided considerably smaller particles with a Z-average diameter of below $400\ \text{nm}$, and of even below $300\ \text{nm}$ in the case of the Gelatin A/Tween20/Span40 system. The results are in general accord with a previous work by the authors,^{10,18} when pure PLA with a M_w of $\sim 35\ 000\ \text{g mol}^{-1}$ was used for preparing submicron particles. Therefore, it was concluded that the presence of Lactoval did not affect the formation of submicron particles. Through observing the distribution of particle sizes (Figure 7), it is clear that although systems containing Tween20 and Span40 produced submicron particles where no fraction exhibited a diameter greater than $1\ \mu\text{m}$, this was in contrast with PluronicF68 examples, where particles approaching $1.5\ \mu\text{m}$ were detected. This information is especially important if degradable particles are considered in living systems. In medical applications various particle sizes can be utilized. Particles possessing a diameter of between $100\ \text{nm}$ and $600\ \text{nm}$ might be useful for targeted drug delivery systems,

Table III. Thermal Properties of PLLA Polycondensates

Lactoval (wt %)	T_c ($^\circ\text{C}$)	dH_c ($\text{J}\cdot\text{g}^{-1}\ \text{K}^{-1}$)	T_{m1} ($^\circ\text{C}$)	T_{m2} ($^\circ\text{C}$)	dH_m ($\text{J}\ \text{g}^{-1}$)	T_g^a ($^\circ\text{C}$)
0.00	n.d.	n.d.	133.8	142.9	24.0	44
0.18	86.5	3.559	119.7	130.4	28.3	42
0.36	88.0	n.d.	93.7	104.8	12.5	38
0.50	n.d.	n.d.	n.d.	n.d.	n.d.	39
0.73	n.d.	n.d.	n.d.	n.d.	n.d.	40

^aValue taken from 2nd heating run. n.d., not detected.

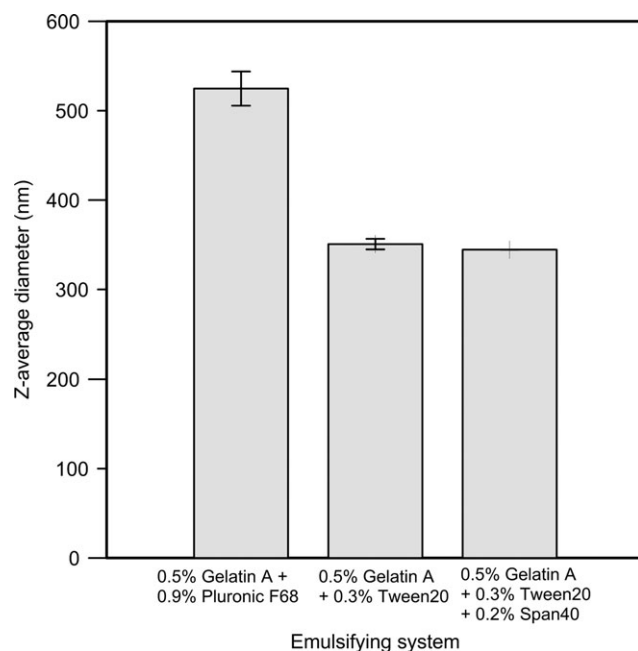


Figure 6. Z-average diameter of particles prepared from material containing 0.5% Lactoval with various emulsifying systems.

whilst particles of approximately 1–3 μm are useful for inhalation drug delivery, and larger particles $<5 \mu\text{m}$ are used in par-parental drug delivery.^{19,20}

The reason of different particle size and distribution obtained with gelatin containing Pluronic F68, Tween20, and Span40 shown in Figures 6 and 7 can be explained through, so called, hydrophilic-lipophilic balance parameter (HLB), which is a

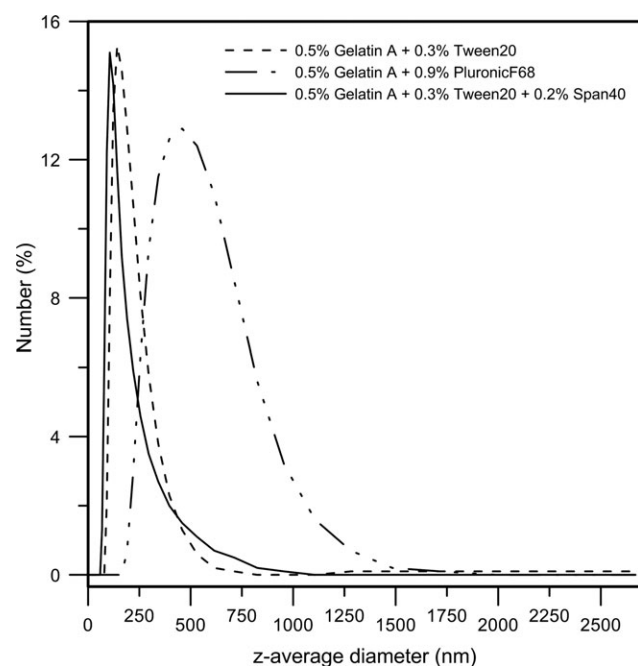


Figure 7. Particle size distribution of prepared systems.

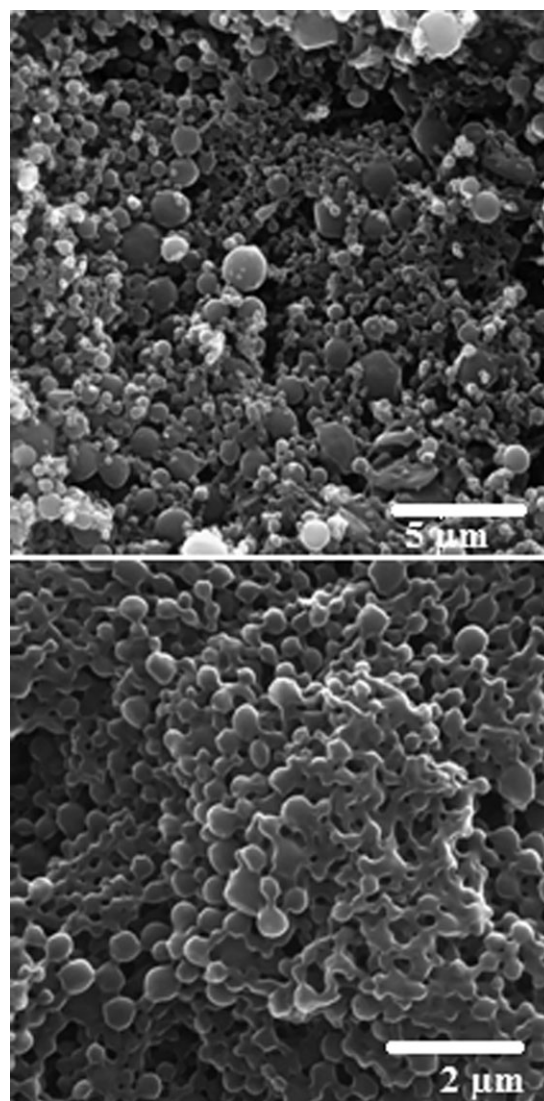


Figure 8. SEM micrographs of prepared submicron particles. Upper - 0.5% Gelatin A and 0.9% Pluronic F68; Lower - 0.5% Gelatin A and 0.3% Tween20.

characteristic of nonionic surfactants. The HLB measures the relative influence of hydrophilic and hydrophobic parts in the surfactant molecule. It is a value between 0 and 60 characterizing the affinity of a surfactant for water or oil. On the basis of our previous work it was stated, that HBL value of 16 was optimal from both particles diameter (low diameters were obtained) and their size distribution width.¹⁴ Because HLB of Pluronic F68 (29) was higher than the other emulsifiers (Tween20 = 16.7; Span40 = 12.7) lower particles diameter and narrow polydispersity could be achieved with these two systems.

Microscopic pictures of the selected systems are depicted in Figure 8. In the upper part, the system emulsifying with 0.5% Gelatin A and 0.9% Pluronic F68 can be seen. It is apparent that this sample contains a mixture of particles of both small and large diameters, as detected by the light scattering method. Conversely, the lower part of Figure 8 shows particles emulsified

with the 0.5% Gelatin A and 0.3% Tween20 emulsifying system, which displayed a considerably lesser diameter ($<1 \mu\text{m}$).

CONCLUSIONS

The goal of this work was to investigate the effect of inorganic impurities in the reaction mixture on melt lactic acid polycondensation, catalyzed by an organo-metallic compound, and the ability of this material to form submicron particles with different emulsifying systems. The calcium-based inorganic compound—Lactoval, derived from actual biotechnological production, was used as the source of the impurities. The results obtained indicated that the inorganic component affects the polycondensation reaction moderately through molecular weight reduction when in a concentration of up to 0.75 wt %. A greater content than this led to total decomposition of the product. However, noticeable changes in thermal behavior were observed. While low concentrations of inorganic impurity caused a significant drop in melting temperatures, concentrations of > 0.5 wt % resulted in highly amorphous products. The material was suitable for forming submicron particles possessing diameters ranging from 280 to 520 nm, and the presence of the impurity had no significant effect on particle shape and dimension.

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