

King Saud University

Arabian Journal of Chemistry

www.ksu.edu.sa



ORIGINAL ARTICLE

Thermal degradation and kinetics stability studies of oil palm (*Elaeis Guineensis*) biomass-derived lignin nanoparticle and its application as an emulsifying agent



Asim Ali Yaqoob^a, Siti Hajar Sekeri^{a,*}, Muhammad Bisyrul Hafi Othman^a, Mohamad Nasir Mohamad Ibrahim^{a,*}, Zahra Hosseinpour Feizi^b

^a Materials Technology Research Group (MaTRec), School of Chemical Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

^b Chemical Engineering Department, Lakehead University, 955 Oliver Road, Thunder Bay, ON P7B 5E1, Canada

Received 18 March 2021; accepted 20 April 2021 Available online 28 April 2021

KEYWORDS

Nano-sized lignin; Emulsion; Emulsifying agent; Empty fruit bunch; Shelf-life; Kinetic stability **Abstract** In this paper, lignin was extracted from oil palm empty fruit branch fibers by using a soda pulping process. The homogenizing method was used to reduce the size of lignin into nanoparticles. Four different homogenization shear speeds *i.e.* 6400, 8400, 10,400, and 12,400 rpm were explored to control the particle size of lignin. Lignin nanoparticles (LNPs) were characterized using FT-IR, UV, TEM, SEM, zeta potential, kinetic thermal degradation, and toxicological analyses. The stability of water-in-oil (W/O) emulsions was characterized by visual observation and cross-polarized microscopy (CPM). The FT-IR results showed similar spectra of all the samples while the particle distribution from TEM demonstrated up to 98% of the samples are in the nano range. The zeta potential of LNP12* (emulsifying agent produced from LNP12 at 12,400 rpm) was observed at |-32.0| mV and found to be the best among all samples to stabilize W/O emulsion at a ratio of 30:70 (water to oil) as a result from creaming index percentage and CPM. Therefore, LNP12 was further analyzed for shelf-life prediction using a kinetic thermal degradation study. Flynn-Wall-Ozawa and Kissinger methods were used in kinetic thermal degradation to determine the activation energy (E_a) of LNP12. It was found that both methods are in good agreement. The E_a value of LNP12 demonstrated better stability and longer shelf-life (around 20 months) at

* Corresponding authors.

Peer review under responsibility of King Saud University.



https://doi.org/10.1016/j.arabjc.2021.103182

1878-5352 © 2021 The Author(s). Published by Elsevier B.V. on behalf of King Saud University.

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

E-mail addresses: asim.yaqoob@student.usm.my (A.A. Yaqoob), hajarsekeri@gmail.com (S.H. Sekeri), bisyrul@usm.my (M.B.H. Othman), mnm@usm.my (M.N.M. Ibrahim).

a temperature below 35 °C. LNP12 showed to be safe both, *in vitro* and *in vivo* toxicological methods at a concentration below 2000 mg/kg.

© 2021 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The current industrial trend has shown a significant interest in changing emulsifiers from animal-based or synthetic chemicals to natural plant-based due to the cheaper production cost, environmentally friendly, and ease to handle. Lignin is the second most naturally abundant biopolymer in plant cell walls (Rashid et al., 2018). It is an amorphous, highly branched polyphenolic macromolecule with a complex structure and high molecular weight as appeared in Fig. 1. Lignin springs up from an enzyme-mediated dehydrogenative polymerization and contains three major phenylpropanoid monomers; p-coumaryl, coniferyl, and sinapyl alcohol (Ibrahim et al., 2004; Hussin et al., 2018). These monolignols lead and link together with carbon-carbon linkage between aliphatic and aromatic bonds. Lignin consists of strong reactive sites of phenolic (highly branched polyphenolic), aliphatic hydroxyl groups (Wei et al., 2012). The amphiphilic property of lignin could make this polymer a potential bio-based candidate to be used as an emulsifying agent where it can reduce the interfacial between oil and water (Sipponen et al., 2017). The adsorption of unmodified lignin is reported to occur at the oil/water interface and develop emulsion stabilization by inducing steric and electrostatic forces between the droplets. Several studies already reported the lignin application as an emulsifying agent as summarized in Table 1. However, no analysis has been performed on revealing the shelf-life stability

of lignin in an emulsion as an emulsifying agent. In the present work, nanosized lignin extracted from oil palm (Elaeis Guineensis) empty fruit branch (EFB) fibers, was used as an emulsifying agent. The four different shear speeds of the homogenizer were carried out to prepare the different nanosized lignin. The prepared nanosized lignin was further analyzed by using the kinetic thermal degradation process to determine the activation energy (E_a) . The E_a indicates the stability and longer shelf-life at various temperature ranges. Results obtained from the kinetic thermal degradation study will ensure the stability of the nanosized lignin in an emulsion which is also performed for the first time in this study. The shelf-life prediction of lignin through the kinetic thermal degradation is critical to be analyzed. It could predict the stability of nanosized lignin in emulsion, which directly affects the performance of nanosized lignin as an emulsifying agent. A fundamental kinetic study could predict the stability and behavior of biomass materials (including lignin) at various temperatures already stated in the literature. For example, Zhou et al. (2015) reported that the derivative thermogravimetric (DTG) curves could be used to determine the kinetic parameters which are more convenient and sensitive to evaluate the kinetic process. Generally, kinetic studies using either isothermal or non-isothermal methods are conducted to extract information about the optimum conditions, mechanism of sorption, and possible rate-controlling step of the samples. The non-isothermal kinetic method is usually performed at varying temperatures, while



Fig. 1 Lignin structure and its monomers

 Table 1
 Previously reported studies on lignin application as an emulsifying agent.

| Main finding | Emulsion | References |
|---|----------------|----------------------|
| | droplets | Kelefellees |
| Produced a stable O/W (water- | ~100 nm | (Silmore |
| cyclohexane) of Pickering | | et al., 2016) |
| emulsions. A relatively small | | |
| amount of emulsifying agent was | | |
| needed to stabilize emulsions. | | |
| Stabilized pH-responsive of O/W | 20–40 µm | (Shi et al., |
| Pickering emulsions. Stabilized the | | 2019) |
| emulsion by adsorbing modified | | |
| lignin on the surface using | | |
| electrostatic interaction. | | |
| Stabilized O/W (kerosene-in-water, | 1–100 µm | (Li et al., |
| 30:70 of ratio) emulsion with low | | 2016) |
| surface activity. | | |
| Stabilized Pickering emulsions are | 115-300 nm | (Bertolo |
| used as encapsulation agents of | (lignin | et al., 2019) |
| curcumin, a polyphenol with a wide | particle) | |
| range of pharmacological | | |
| applications. | | |
| Stabilized O/W emulsions with a | 0.717 μm | (Czaikoski |
| low concentration of lignin. | | et al., 2020) |
| Cytotoxicity of lignin samples | | |
| towards HT-29 and Caco-2 cells | | |
| showed a dose-response | | |
| relationship, determining non- | | |
| cytotoxic concentrations for the | | |
| emulsifying agent that can produce | | |
| stable emulsions. | | |
| Stabilized Pickering emulsions | 259 nm (lignin | (Sipponen |
| (toluene-in-water). | particle) | et al., 2017) |
| Used lignin particles for drug | ~220 nm | (Figueiredo |
| delivery and enhanced | | et al., 2017) |
| antiproliferation effect in cancer | | |
| cells and biomedical applications. | | 0.7 |
| Synthesized lignin from (W/O) | ~90 nm to | (Nypelo |
| microemulsions and used it in the | 1 mm | et al., 2015) |
| interfacial stabilization of Pickering | | |
| systems and organic carriers for | | |
| silver metal. | 227 404 | (0) (1) |
| Revealed the application of light | 237–404 nm | (Qian et al., 2014) |
| particles as suffactants for CO_2/N_2 | | 2014) |
| amulaified) Pickering amulaions | | |
| Promoted high value bio additive | 105 107 nm | (Tian et al |
| for multifunctional | 195–197 IIII | (11all et al., 2017) |
| nanocomposites. The enriched | | 2017) |
| nhenolic hydroxyl groups of lignin | | |
| provided good interfacial adhesion | | |
| and also enabled it to be | | |
| compatible with nonpolar polymer | | |
| matrix. | | |

the isothermal method is conducted at a constant temperature (Cortés and Bridgwater, 2015). The temperature could also significantly affect the stability of emulsions by either the stability of the particle size (emulsifying agent and emulsion) or the speed of the emulsifying process at its thermodynamic equilibrium. Sapei and Damayanti (2017) found that the effects of ingredient formulations, process parameters, and storage temperatures on the emulsion shelf-life could be simply predicted from the kinetics studies. The shelf-life of lignin nanoparticles (LNP) also plays a critical role in its performance as an emulsifying agent since it is expected to be directly proportional to its degradation with temperature and biocompatibility with emulsion. The present research aimed to produce stable lignin nanoparticles from oil palm EFB and use them as bio-based emulsifying agents for the water-in-oil (W/O) emulsions. Lowering the particle size is believed to increase the surface area and kinetically stabilize the emulsion (Hattalli et al., 2002). Generally, the emulsifying agent is used to reduce the attractive forces that occur naturally between the molecules of the same liquid and hamper their aggregation (Beisl et al., 2017). Nano-emulsions are introduced as emulsions containing nanosized particles where the size of droplets falls at the range of 10-100 nm (Hattalli et al., 2002). Nanoemulsions are kinetically stable since they are generally thermodynamically stable. Therefore, the kinetic study is necessary to be conducted to prove this statement when nanosized lignin is used as an emulsifying agent. The toxicity test (both in vivo and in vitro) has also been performed to ensure the safeness of the produced lignin-based emulsifying agent when applied in food and biomedical applications.

2. Experimental detail

2.1. Materials

The raw material of the oil palm empty fruit bunch (OPEFB) was supplied by Sabutek (M) Sdn. Bhd. Cooking oil was brought from a local market (Saji, a Malaysian brand), Sulphuric acid (H₂SO₄, 95–97%), sodium hydroxide pellet (NaOH), and n-pentane (99%) were from QRec, Malaysia. 1,4–Dioxane was from HmbG Chemicals, Germany and acetic acid (CH₃COOH) was from Chem AR Systerm, Malaysia.

2.2. Extraction method

Soda lignin (SL) was extracted from black liquor of the OPEFB fiber pulping process at the biomass to tap water ratio of 1:8 (w/w) and mixed with 30% (w/w) of NaOH in a 10 L stainless steel rotary digester for 3 h at 170 °C. The soda lignin was precipitated from the black liquor using 20% v/v H₂SO₄ until pH was reduced to 2. Subsequently, the mixture was centrifuged at 3500 rpm for 10 min to remove the excess aqueous phase and collect the concentrated SL before drying in the oven at 50 °C for 3–4 days. The dried SL was then ground to a powder form and purified following the published method by Ibrahim et al. (2011). In brief, the purification method of SL involved refluxing via Soxhlet apparatus filled with 99% n-pentane for 6 h at 37 °C to remove lipophilic non-lignin matters such as wax and lipids.

2.3. Preparation of lignin nanoparticle (LNP)

LNPs were prepared using a mechanical homogenizer, IKA T25 digital ULTRA-TURRAX, USA with IKA dispersion tool S 25 N-25F. Around 4 g of SL was soaked and dispersed in 400 mL deionized water. The lignin dispersion was treated at four different shear speeds of the homogenizer for 1 h (for each speed). Lignin samples treated at 6400, 8400, 10,400, and 12,400 rpm/1h were labeled as LNP6, LNP8, LNP10, and LNP12, respectively. Previous research suggested the choice of shear speeds of the homogenizer of 6400, 8400, 10,400, 10,400, and 12,400 rpm/1h for this experiment. It saves both time and resources (Sekeri et al., 2020). Thereafter, the samples were dried using a freeze-dried method at a temperature around -45 °C.

2.4. Emulsifying agent and emulsion preparations

In the preparation of the emulsifying agent, around 2 g $(2000 \text{ mg mL}^{-1})$ of each lignin sample (SL, LNP6, LNP8, LNP10, and LNP12) was dissolved in 10 mL of NaOH (1 M). Then, 30 mL of distilled water was added to each sample, and its pH was adjusted to 3.5 by adding a few drops of acetic acid (CH₃COOH). The solutions were then left for particle coagulation for 10 min. In preparing the emulsions, 2% (40 mg mL⁻¹) of the concentration of the emulsifying agents (SL*, LNP6*, LNP8*, LNP10*, and LNP12*) were used relative to the total volume percentage of the oil and water (v/v). Cooking oil and distilled water were used to form a W/O emulsion at a ratio of 30:70 (v/v), water to oil, and homogenized at the speed of 10,400 rpm for one min. The emulsifying agent in the amount of 2% (v/v) was then added to each of the prepared emulsion and homogenized at 10,400 rpm for another one min. The newly formed emulsions were stored in plastic bottles at room temperature for the creaming stability observations.

2.5. Characterization

Fourier transform infrared (FT-IR) spectroscopy analysis was performed on SL, LNP6, LNP8, LNP10, and LNP12 using the Perkin Elmer model System 2000; Norwalk, CT, USA. FT-IR is a fast-analytical technique to identify functional groups of the samples. The analyses were conducted at the frequency range of 4000 cm⁻¹ to 600 cm⁻¹ with a resolution of 4 cm⁻¹ and 16 scans. The functional groups of the samples were determined using KBr technique at a ratio of 1:100 (w/w).

The ultraviolet–visible spectroscopy (UV–vis) analysis of SL before and after being nanosized was conducted by using Shimadzu UV–Vis 2600 Kyoto, Japan Spectrophotometer. For this analysis, 6 mg of each sample was dissolved in 10 mL ofdioxane–water solution (9:1, v/v). Then, 1 mL of the aliquot was diluted in 10 mL of 50% 1,4-dioxane. The samples were measured between 250 and 400 nm.

The transmission electron microscope (TEM-Model Zeiss Libra 120; Jena, Germany) was used to characterize LNPs (LNP6, LNP8, LNP10, and LNP12). Detailed images of the samples were captured, and the size of the particles was measured. The samples were dispersed in an adequate amount of deionized water, then a drop of the top layer was placed on a copper grid. Samples were incubated for a couple of min and filter paper was used to remove the excess water before the images were captured.

The morphology of SL before and after being nanosized was observed by images of SEM using the extreme high-resolution field emission scanning electron microscope (SEM-Quanta FEG 650, Fei; Columbia, MO, USA). The SL and LNP samples were sputter-coated with gold and the magnification of the images was set at $100,000\times$.

Zeta potential of SL, LNP6, LNP8, LNP10, and LNP12 was measured using a zeta sizer (Malvern Instrument, Model Nano-25, USA). About, 5 mL of 0.1 mg mL⁻¹ of each lignin sample was dispersed in deionized water before filling the DTS 1070 cuvette for the zeta potential measurement. Each sample took approximately 2 min for stable data.

The emulsions were left for 7 days at room temperature for phase separation by gravitational force. For stability analysis, emulsions containing emulsifying agents of SL, LNP6, LNP8, LNP10, and LNP12 were placed in translucent bottles. Upon completion of 7 days period, the height of what and the boundary layers were measured. The images of emulsions were also taken before and after 7 days for comparison.

The images of sizes and shapes of emulsion droplets of SL, LNP6, LNP8, LNP10, and LNP12 were studied using polarized light microscopy (Olympus, BX53, F.CCD, UK). This technique uses polarized light to enhance the contrast and improve the image quality that is visible primarily due to their optically anisotropic character. Samples of emulsions were taken out using a plastic pipette and placed on a clean and dry microscope slide. The images of emulsion droplets were captured under the light with 10x magnifications.

After characterizations of all samples, the most stable LNP will be chosen to further analyze for kinetic thermal degradation. The non-isothermal thermogravimetric analysis (TGA) was conducted using TGA-Perkin Elmer Thermal Analyzer, model TGA/SDTA 851; Akron, OH, USA. The sample (5 mg) was weighed and placed in a platinum pan. The temperature was set at 30-800 °C with four different heating rates (B) at 5, 10, 15, and 20 °C min⁻¹ in a nitrogen atmosphere. The mass loss versus temperature was plotted. The onset decomposition temperature (T_{onset}), maximum temperature (T_{max}), end temperature (T_{end}), and residual mass (R_w) at 800 °C were determined. The kinetic degradation was evaluated from TGA data using Flynn-Wall-Ozawa and Kissinger methods at various heating rates of 5, 10, 15, and 20 °C min⁻¹. The shelf-life of LNPs was predicted at varied temperatures. Theoretically, the rate of reaction by thermogravimetric analysis is defined as the ratio of the loss from actual mass at the time (t) to the loss from total mass at complete process degradation as represented by Eq. (1) below. All related equations and theories were extracted from Othman et al. (2016b) where α , M₀, M_{f} , and M_{f} is a rate of conversion, the initial mass of the sample, the mass of sample at time t, and final mass of the sample, respectively. The rate of conversion (α) is directly proportional to the concentration of the sample.

$$\alpha = \frac{M_o - M_t}{M_o - M_f} \tag{1}$$

Constant rate, k is given by the Arrhenius equation as presented below (Eq. (2)).

$$k = Ae^{\left(-\frac{L_a}{RT}\right)} \tag{2}$$

where A is representing the frequency factor (s^{-1}) , E_a is the activation energy of degradation reaction (kJ mol⁻¹), R is a gas constant and T is the absolute temperature.

The value of E_a can be determined by using several methods such as F–W– O, Kissinger, C–Red, H–Mat, Mac–T, and van Krevelen. The previous study by Othman et al. (2016a) reported that the F–W–O and Kissinger methods are preferred for dynamic heating experiments and have been chosen for further discussion as these are the effective methods to evaluate the dependency of E_a on α . From Eq. (3), the graph of log β versus 1000/T was plotted at different conversion percentages. The linear lines from the graph were used to determine the E_a by substituted slope values into Eq. (4).

$$\operatorname{Log}\beta = \log \frac{AE_a}{g(\alpha)R} - 2.315 - \frac{0.457E_a}{RT}$$
(3)

$$E_a = \frac{slope \times R}{0.457} \tag{4}$$

Meanwhile, the Kissinger method calculates the E_a value that is derived from Eq. (5). From Eq. (5), *n* is the reaction order, T_p is the absolute temperature and α_p is the mass loss at the maximum mass loss rate $(d\alpha/dt)_p$.

$$\ln\frac{\beta}{T_p^2} = \ln\frac{\mathbf{AR}}{\mathbf{E}_a} + \ln\left[n(1-\alpha_p)^{n-1}\right] - \frac{\mathbf{E}_a}{\mathbf{R}T_p}$$
(5)

Then, the shelf-life prediction was calculated using Eq. (6), at varying temperatures. The reaction order (*n*) was obtained from the symmetrical index of the DTG peak as presented in Eq. (7). The value of $[d^2\alpha/dt^2]_L$ and $[d^2\alpha/dt^2]_R$ was found from the second derivative thermogravimetric (DDTG) curve of each heating rate corresponds to the left peak and the right peak, respectively. The value of A was calculated using Eq. (2).

$$t_f = \frac{(1 - 0.95^{1-n})}{A(1-n)} e^{\frac{E_d}{RT}} (n \neq 1)$$
(6)

$$n = 1.88 \frac{\left[\frac{d^2 \alpha}{dt^2}\right]_L}{\left[\frac{d^2 \alpha}{dt^2}\right]_R} \tag{7}$$

The most stable of LNP also was chosen for further testing the toxicology properties in vitro and in vivo toxicity studies which were conducted on rats by feeding the formulation orally following the Guidelines laid by the Organisation for Economic Co-operation and Development (OECD), International Animal Ethical Regulatory Body. The in vitro biocompatibility test of the extracted lignin was tested on a human umbilical vein cell line (ea.hy926) to evaluate the potential cytotoxic effect of lignin using the 3-(4,5-dimethylthiazol-2-y 1)-2,5-diphenyl tetrazolium bromide (MTT) assay. The assay was carried out using the method described previously with minor modification by Ahamed et al. (2012). Experimental procedure and handling of animals were carried out according to the guidelines set by the International Forum for Good Animal Welfare Practices, World Organisation for Animal Health and Welfare Society, and the Organisation for Economic Cooperation and Development (OECD) testing guidelines.

3. Results and discussion

3.1. FT-IR analysis

The functional groups of SL and LNPs were determined using FT-IR analysis as shown in Fig. 2a. The wide absorption band of hydroxyl groups or phenolic compounds was found at 3407–3402 cm⁻¹. The C-H stretching of methyl or methylene groups was observed around 2935-2910 cm⁻¹. Whereas a broad medium band observed at 1707-1700 cm⁻¹ revealed a conjugated carbonyl stretching. Furthermore, the peak at range 1512–1510 cm⁻¹ defines characteristic of lignin aromatic rings resulted from aromatic skeletal vibration, and the bands observed at 1134-1128 cm⁻¹ due to the ether stretching (syringyl and guaiacyl) (Delgado et al., 2019). Ibrahim et al. (2011) proposed that the appearance of guaiacyl unit in lignin activates the site of polymerization. Thus, the structure of lignin could develop chemical and biological interactions which could be a preferable base for the expansion of bio-based products such as an emulsifying agent (Lievonen et al., 2016). As



Fig. 2 (a) FT-IR spectra of SL and LNPs, and (b) UV–Vis spectra of SL and LNPs.

reflected in the FT-IR results, SL and LNPs samples showed very similar spectra, revealing that converting SL to LNPs did not affect lignin functional groups. This was also supported by Rahman et al. (2018) that the functional groups of nanosized lignin were found to be similar to the pristine lignin.

3.2. UV-visible spectra analysis

Production of LNP as an emulsifying agent and its application in industries such as food, personal care, and cosmetics requires a product free from any impurities. This is to avoid undesired changes in the texture, taste, and stability of emulsions. To address this crucial factor, after the purification process of lignin, the particles were analyzed using UV-vis spectroscopy to guarantee all impurities such as degraded polysaccharides, wax, and lipids were eliminated before further characterizations (Ibrahim et al., 2004; Lee et al., 2013). The purity of lignin could be determined according to its excessive number of phenols from the absorption at the wavelength of 280 nm. Interestingly, as shown in Fig. 2b, LNP12 exhibited the purest lignin followed by LNP10, LNP 6, LNP8, and SL. This can be concluded that the purity of lignin improved after the nanosizing process.

Besides, it is revealed by Ibrahim et al. (2011) that the UVvis analysis can be used to discover the phenolic content since it is the most significant functional group in the lignin structure. As seen in Fig. 2b, all samples depicted two maximum absorption peaks: the first one around 230-245 nm and the second one around 270-280 nm. As reported by Sun et al. (1999) and Hattalli et al. (2002) the UV-vis absorption of lignin at 230 nm and 290-300 nm is originated from nonconjugated ionized phenolic groups and conjugated structural non-ionized elements, respectively. Also, Abdelaziz and Hulteberg (2017) revealed the absorption at the wavelength of 280 nm to be due to aromatic rings/non-conjugated phenolic groups. The wavelength at 273, 280, and 313 nm is ascribed to lignin monomers of syringyl (S), guaiacol (G), and phydroxyphenol (H), respectively (Singh and Dhepe, 2016). Hence, it could be concluded from the obtained UV-vis results that SL and LNPs mainly consisted of G units.

3.3. TEM analysis

Lignin is normally a macro-sized polymer. Since the size of nanostructured materials is generally in the range of 1–100 nm, they could offer unique properties due to their increased surface area. Thus, LNPs prepared in this study were mechanically treated with a high shear homogenizer to have a diameter of less than 100 nm. This method has been previously used by Matsakas et al. (2018) and Nair et al. (2014) where Nair et al. (2014) used high shear homogenization at

15,000 rpm for an hour to produce nanosized lignin. However, only 19% of the produced particles had a size of below 100 nm and thus, the treatment period was required to be extended to four hours to produce lignin nanoparticles. Fig. 3 shows the histogram of the size distribution of LNPs mechanically sheared at different shear speeds. The size distribution of LNP6, LNP8, and LNP10 shows more than 97% of the particles in the nano range (<100 nm). Meanwhile, increasing the shear speed of the homogenizer to 12,400 rpm resulted in completely having a diameter less that than 100 nm.

As shown in Fig. 4, the majority size of the produced LNPs was around 100 nm. However, the particles of LNP6 and LNP8 seemed to be agglomerated and overlapped, which might further affect the stability of the emulsions. By increasing the speed of the homogenizer, LNP10 particles were dissipated. This led to an effective reduction in the particle size and further concluded that LNPs with the size of 20-100 nm could be produced using the high-shear homogenizer at the speed of 12,400 rpm. The differences of the series were also observed at the particle dispersion and distribution. The LNP 12 series have immense particle dispersion compared to other samples. The emulsion can remain stable for a long period if the hydrophobicity interaction ability of the nanoparticles is high, which further leads to their more adsorption to the surface of oil droplets and thus, prevent emulsion coalescence. Therefore, lowering the particle size created a large surface area which improved the hydrophobicity of the nanoparticles and increased their active surface to stabilize the emulsion.

3.4. Scanning electron microscopy (SEM) analysis

The surface morphologies of SL and LNPs were characterized by SEM analysis and the images are shown in Fig. 5. The SEM images showed a rough surface with overlapped large particles



Fig. 3 Histogram of the size distribution of LNPs mechanically sheared at different shear speeds: (a) 6400 rpm, (b) 8400 rpm, (c) 10,400 rpm and (d) 12,400 rpm.





Fig. 4 Transmission Electron Microscopy (TEM) images of (a) LNP6, (b) LNP8, (c) LNP10, and (d) LNP12.



Fig. 5 Scanning electron microscopy (SEM) images of (a) SL, (b) LNP6, (c) LNP8, (d) LNP10, and (e) LNP12.

of SL. Azimvand et al. (2018) reported that micro-size lignin had a flat surface morphology as also been observed in Fig. 5a. SL particles were heterogenized as the size is relatively larger and microparticles are in agglomerated form.

As seen in Fig. 5b–e, reducing the size of lignin to nanosize led to the appearance of smaller particles in all images taken from LNPs. Interestingly, LNP12 formed colloidal lignin particles as supported by the TEM image where it showed more uniformly distributed (not overlapped) particles compared to other LNPs. As reported by Bai et al. (2018), LNPs can offer smooth surfaces for the formation of thin films and therefore produce stabilized emulsions (Henn and Mattinen, 2019). Hence, nanosize particles (particularly LNP 12) can act as good emulsifying agents.

3.5. Zeta potential analysis

Zeta potential is defined as the difference in potential between the surface of a tightly bound layer of ions on the particle surface which affects the behavior and properties of colloidal suspensions. The charge of the surface of a polymer or nanoparticles can directly affect the zeta potential values (Zhang et al., 2008). In this work, the zeta potential was used to estimate the colloidal stability of the particles since the particle stability directly affects the emulsion stability. Fig. 6 shows the zeta potential graphs with three curves as the average value of zeta potential for SL, LNP6, LNP8, LNP10, and LNP12 samples. Generally, lignin is a negatively charged compound due to its phenolic hydroxyl and carboxyl functional groups (Fig. 2a and b), hence lignin has depicted a negative zeta potential in this study. Considering that a smooth zeta potential peak represents the accuracy of the results, by comparing the graphs in Fig. 6, it was found that only LNP12 showed a smooth curve and a nice peak. While others showed peaks in irregular shapes and rough side-lines which may lead to a varied colloidal behavior of the nanoparticles than expected, which was further evaluated in this work. The average zeta potential values and lignin solution stability behavior were shown in Table 2. The zeta potential obtained for SL, LNP6, LNP8, LNP 10, and LNP12 was measured to be |-39.0, |-29.2, |-25.0, |-26.0, and |-32.0 mV, respectively, which directly affected their stability. Tong et al. (2018) reported that the value of zeta potential lower than $|\pm$ 30 mV | manifested signs of instability due to insufficient electrostatic repulsion between particles to avoid agglomeration. The stability of an emulsion is guaranteed when the repulsive force between the particles in a suspension or droplets in an emulsion exceeds the attractive force (Gupta and Washburn, 2014). Thus, a zeta potential value above or equal to \pm 30 mV leads to a stable suspension or emulsion since a repulsive force is generated between the substrates which hinders their agglomeration or coalescence.

Based on the obtained results, LNP12 was recorded to be the most stable among LNPs suspension. Although the obtained zeta potential value was higher for SL than LNP12, this study intended to formulate kinetically and thermodynamically stable LNPs to be used as emulsifying agents. The lower zeta potential values of LNP6, LNP8, and LNP10 suspensions had also led to lower suspension stability compared to LNP12. The effect of the lower zeta potential values of these samples is also observed in the TEM images as LNP6, LNP8 and LNP10



Fig. 6 Zeta potential graphs of (a) SL, (b) LNP6, (c) LNP8, (d) LNP10, and (e) LNP12.

showed more agglomerated particles with non-uniform distribution compared to LNP12. The results show that LNP6, LNP8, and LNP10 have less potential to be effective emulsifying agents compared to LNP12.

3.6. Emulsion stability

The stability of emulsion refers to the ability to stay in its properties since emulsions have the propensity to break down after a passage of time. In previous work (Sekeri et al., 2020), it was

| Table 2 LNPs. | Zeta potential values and | the stability behavior of |
|------------------|---------------------------|---------------------------|
| Sample | Zeta Potential (mV) | Stability behavior |

| Sample | Zeta Potentiai (IIIV) | Stability beliavior |
|--------|-----------------------|---------------------|
| SL | -39.0 | Stable |
| LNP 6 | -29.2 | Not Stable |
| LNP 8 | -25.0 | Not Stable |
| LNP 10 | -26.0 | Not Stable |
| LNP 12 | -32.0 | Stable |

found that 2% (w/v) of LNPs emulsifying agents was the optimum dosage to produce good stability in the W/O emulsion. Hence, 2% (w/v) of the samples were used in the W/O emulsion to evaluate the emulsifying effectiveness as shown in Fig. 7. The top images of Fig. 7 illustrate W/O emulsions containing SL*, LNP6*, LNP8*, LNP10^{*} and LNP12* emulsifying agents right after the preparation of emulsion samples. While the bottom images are the emulsions after being stored for 30 days. During the storage period, the emulsion tends to break down and separate, which depends on the density and distribution between the droplet and the medium. Flocculation, creaming, sedimentation, aggregation, coalescence, and Ostwald are the phenomena involved in the instability of emulsions.

As depicted in Fig. 7, emulsions containing SL*, LNP6*, LNP8^{*}, and LNP10* showed instability due to the formation of three layers of liquid phase after 30 days. The top layer is the oil phase, the middle layer is the emulsion phase and the bottom layer is the water phase. However, emulsions containing LNP6* and LNP10* seemed slightly different as they both formed a thinner layer of the oil phase and a thicker emulsion phase compared to SL* and LNP8*. Meanwhile, the emulsion containing LNP12* can be considered as the most stable emulsion as it exhibited only two layers *i.e.* the emulsion phase at the top and the water phase at the bottom. The stability of

emulsion generated by using LNP12* could be due to the smallest nanoparticle size of this sample compared to others. The reason may be attributed to that un-homogenized particles tend to fall into Ostwald ripening, creaming, and coalescence due to the increased particle and droplet size in emulsions (Li et al., 2016). The results revealed that the phase separation of the emulsion occurred since the utilized emulsifying agents had varied particle sizes (Xin et al., 2013). As seen in the TEM and SEM images, as well as the zeta potential analysis, it can be concluded that LNP12* with the most homogenized and the smallest sized nanoparticles formed the most stable emulsion.

3.7. Cross-polarized microscopy analysis

The droplet size of emulsions was studied by using crosspolarized microscopy (CPM). The images of prepared emulsions (denoted as SL**, LNP6**, LNP8**, LNP10**, and LNP12^{**}) shown in Fig. 8 were taken by microscope after being stored for 30 days. The CPM images of SL**, LNP8**, and LNP10** emulsions showed slurry emulsions where the emulsion droplets mostly did not have specific shapes. These images also proved the instability of emulsions containing these emulsifying agents. Meanwhile, emulsions containing LNP6* and LNP12* showed more spherical droplets, while the sizes were varied. The droplet size of emulsion containing LNP6* was larger than that of containing LNP12* and tended to break down within the time. The emulsions used in this study were formed using high shear homogenizer which breaks large droplets into small droplets due to strong turbulence and hydraulic shear (Gupta et al., 2016). However, the small droplets of LNP6**, LNP8** and LNP10** shown in Fig. 8 tended to coalescence after the process and formed partial emulsification of the oil. Coalescence can be prevented by the full protection of the interfacial area by the particles (in our case LNP12)



Fig. 7 The fresh W/O emulsions (top image) and after 30 days (bottom image) when using (a) SL^* (b) $LNP6^*$ (c) $LNP8^*$ (d) $LNP10^*$ and (e) $LNP12^*$ as the emulsifying agents.



Fig. 8 The cross polarized microscopy image (CPM) of (a) SL^{**} (b) LNP6^{**} (c) LNP8^{**} (d) LNP10^{**} and (e) LNP12^{**}.

which leads to a more stable emulsion (Salerno et al., 2016). As observed, using LNP12* as the emulsifying agent promoted a smaller size of emulsion droplets having uniform size and shape, which remained stable over 30 days.

3.8. Kinetic thermal degradation

Thermogravimetric analysis reveals valuable information for understanding different thermal events correlated with materials especially in the preparation of final products. In this study, TGA analysis was used to investigate the effect of changing the lignin particle size (from micro-size to nano-size) on its thermal degradation process. Yaqoob et al. (2020) supported that nanoparticles have strong thermal stability due to their unique properties and small dimension. Since LNP12* showed the best emulsifying performance, it was chosen to be tested for kinetic thermal degradation to predict its shelf-life stability at varying temperatures. The TGA curves depicted in Fig. 9a presents the LNP12 at various heating rates of 5, 10, 15, and 20 °C min⁻¹ at the temperature range of 30–800 °C in the nitrogen atmosphere. The data were extracted and tabulated in Table 3. The thermal decomposition of LNP12 occurred at two stages of degradation. The first degradation occurred at the temperature range of 30-130 °C corresponding to the evaporation of moisture content and excess solvent (< 3.4-4.2% from the overall mass) (Sun et al., 2001). Also, the second degradation occurred at the temperature range of 150-800 °C which could be attributed to the decomposition of hemicellulose part linked to the lignin structure, as well as the fragmentation of inter-unit linkage between the carbonyl and phenolic groups (<61.7-68.3% from the remaining mass) (Chen et al., 2019). The maximum mass loss (DTG_{max}) of LNP12 occurred at the temperature range of 370-400 °C (Fig. 9b) was shifted higher with decreasing β . According to Othman et al. (2016b), this is because of the production of higher thermal stress at high temperatures due to the limited time to achieve the thermodynamic equilibrium. Also, the value of β affects the thermal decomposition, as a higher value of β induces a fast degradation and reduces the amount of maximum mass loss. As known, the higher the heating rate, the faster the weight loss equilibrium is achieved (Rousseau, 2000). The previous studies have found that the thermal stability of lignin can be varied because of its complex structure containing oxygenated functional groups and the associated bonds can occur at different ranges of temperatures (Skreiberg et al., 2011). Hence, during the process, the thermal equilibrium was achieved at low β which reveals the lowest residue obtained at 5 °C min⁻¹ heating rate.

Thermogravimetric analysis (TGA) data was used to analyze the thermal stability and kinetic behavior of LNP12. Skreiberg et al. (2011) reported that the thermodynamically unstable emulsion systems can be stabilized by the improvement of their kinetic stability. Thus, the kinetic thermal stability was used to calculate the E_a , which is the minimum energy required for LNP12* to break and form bonds during the chemical reactions. Since the F-W-O and Kissinger methods have been preferred for dynamic heating experiments, the E_a of the LNP12 thermal degradation was determined using Flynn-Wall-Ozawa (F-W-O) and Kissinger models to interpret data obtained from different heating rates.

3.9. Flynn-Wall-Ozawa (F-W-O) method

The linear lines from the graph shown in Fig. 10a were plotted using Eq (3) as mentioned earlier. The graph resulted in nearly parallel lines due to Doyle approximation (Othman et al., 2015). The E_a values tabulated in Table 4 were calculated using Eq. (4) at conversion percentage (α) between 5% and 30%. The highest E_a value calculated was around 174 (kJ mol⁻¹) which was obtained at 30% conversion and decreased as the conversion percentage is decreased. Based on previous studies, Bove et al. (2019) reported the E_a of lignin to be 130 kJ mol⁻¹ when using this F-W-O method, while Poletto (2017) found that the E_a value at the 10–30% conversion is in the range of 77– 271 kJ mol⁻¹. The obtained values in the present study are in the range as presented in Table 5. The values of E_a were then plotted versus conversion (α) with mass loss ranging from 10% to 90%. Based on Fig. 10b, the E_a values increased from



Fig. 9 (a) Thermogravimetric (TGA) and (b) derivative thermogravimetric (DTG) curves of LNP12 at different heating rates (β).

conversion $\alpha = 0.1$ to $\alpha = 0.7$ and achieved the highest value at $\alpha = 0.7$. The E_a value was then dropped at $\alpha = 0.8$ and continued to fall until $\alpha = 0.9$. These phenomena occurred since E_a depends on the amount of energy required to decompose the material, break and form the molecular chain. Thus, starting from $\alpha = 0.1$ to $\alpha = 0.7$, high energy was required during the modification of the molecular chain and therefore the E_a increased.

3.10. Kissinger method

The Kissinger method is one of the preferred methods used to determine the E_a for the second kinetic degradation (Wellen and Canedo, 2014). The E_a can be computed for kinetic works with less error (<5%) and no dependency on the reaction mechanism. It was supported in the study by Othman et al.

| NSL | β | The residue (%) | T _{onset} (°C) | T _{max} (°C) | T _{end} (°C) | ΔT (°C) |
|-----|--|--|---|--|---------------------------------------|---|
| 12 | 5 | 28.27 | 189.96 | 392.10 | 621.12 | 435.25 |
| | 10 | 28.84 | 190.63 | 381.59 | 617.73 | 437.29 |
| | 15 | 34.11 | 200.62 | 379.96 | 601.52 | 427.96 |
| | 20 | 34.14 | 201.18 | 372.42 | 596.81 | 425.94 |
| | | 1.4 1.6 1.8 2.0 100/T (K ⁻¹) (a) | (d) con] | 1.2 1.4 1.6 100/T (K ⁻¹) (c) | , , , , , , , , , , , , , , , , , , , | 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 ★ 0.9 |
| | 900 800 700 500 400 500 0 0 0 0 0 0 0 0 0 0 0 0 | 2 0.4 0.6 0.8 1.0 Conversion, α (%) (b) | () 900 - 800 - 700 - () 600 - 100 - 200 - 100 - 0 - 0 0 - | 0.2 0.4 0.6 Conversion, α (% (d) | | |

Fig. 10 (a) Graph of log β versus 1000/T following to the F-W-O method of LNP12 with mass loss ranging from 10% to 90% of conversion ($\alpha \times 10^{-3}$), (b) Activation energy, Ea obtained from F-W-O versus conversion α , with mass loss ranging 10% to 90% conversion in nitrogen, (c) Graph of ln (β/T_p^2) versus 1000/T following to the Kissinger method of LNP12 with mass loss ranging from 10% to 90% of conversion ($\alpha \times 10^{-3}$), (d) Activation energy, Ea, obtained from Kissinger method versus conversion α , with mass loss ranging from 10% to 90% conversion in nitrogen.

(2016a) that this method generates highly reliable E_a values within the error mentioned above. The value of E_a was calculated from the slope of the straight-line $ln(\beta/T_p^2)$ versus $1/T_p$

 Table 3
 TGA traces of LNP12 in the nitrogen atmosphere.

| Table 4Activation energy $\alpha < 30$ using the F-W-O m | gy of LNP12 at the c nethod. | onversion |
|--|---------------------------------|----------------|
| Conversion, α (%) | $E_a (kJ mol^{-1})$ | R ² |
| 5 | 90.44 | 0.76 |
| 10 | 111.65 | 0.86 |
| 15 | 123.56 | 0.88 |
| 20 | 137.97 | 0.88 |
| 25 | 155.54 | 0.87 |
| 30 | 174.63 | 0.88 |

from Eq. (5) and plotted in Fig. 10c. Then, the calculated values of E_a were plotted versus α with conversion ranging from 10% to 90% as shown in Fig. 10d. The highest E_a value calculated was around 836 (kJ mol⁻¹) which was obtained at 70% conversion. Based on Fig. 10, the comparison of E_a values from both *iso*-conversional methods showed similar trends in which the E_a increased at 10–70% conversion but decreased at 80–90% conversion.

According to Doyle's approximation method, Table 6 shows the comparison of E_a values between F-W-O and Kissinger methods at low conversion ($\alpha > 30\%$). The F-W-O method represented a higher value of E_a compared to Kissinger. This is because the ln β in Kissinger is related to the function of temperature while log β in the F-W-O method is independent of the temperature (Othman et al., 2016a). Table 6

| Activation energy of lignin (kJ mol ⁻¹) | Kinetic method | Heating rate (°C min ⁻¹) | References |
|--|--|--|---------------------------------|
| 18–588 | Peak analysis-least square method (PA- LSM) | 10 | (Zhou et al., 2015) |
| 67.62 | Arrhenius equation | 80 | (Pasangulapati et al., 2012) |
| 159.39– 174.19 | Non-isothermal multi- Gaussian distributed activation energy model (Gaussian- DAEM-reaction) | 10 | (Zhang et al., 2014) |
| 158-170 | Single Gaussian- DAEM-reaction model | 5, 10 and 15 | (Mani et al., 2009) |
| 37.58 | Isothermal kinetics of torrefaction (Obey Arrhenius equation) | 20 | (Chen and Kuo, 2011) |
| 130–175 | Kissinger method | 2–200 | (Jiang et al., 2010) |
| 105 | Friedman | 2, 5, 10 | (Bove et al., |
| 130 | Flynn-Wall-Ozawa | and 20 | 2019) |
| 123 | Kissinger | | |
| 102 | Vyazovkin | | |
| 77–1537 | Flynn-Wall-Ozawa | 5, 10, 20 and 40 | (Poletto, 2017) |
| 90–174 118–173 (at the conversion of 5–30%) | Flynn-Wall-Ozawa Kissinger | 5, 10, 15 and 20 | Present study |

Table 5Summary of the activation energy of lignin at variousmethods.

shows that a lower E_a value was obtained for LNP12 compared to other lignin types from previous studies (depicted in Table 5). This might be due to the varied particle sizes (of nano, micro, and macro) in different lignin samples which affected the E_a values. Based on the E_a values calculated from these two methods, the constant rate was reliable and obeyed the Arrhenius equation. The similar patterns and trends

Table 6 Activation energy of LNP12 using F-W-O andKissinger methods at varied conversions.

| Conversion, α | E _a (kJ mo | l ⁻¹) | $\Delta E_a \; (kJ \; mol^{-1})$ |
|----------------------|-----------------------|-------------------|----------------------------------|
| | F-W-O | Kissinger | |
| 0.1 | 111.65 | 108.94 | 2.71 |
| 0.2 | 137.97 | 135.91 | 2.06 |
| 0.3 | 174.63 | 173.90 | 0.73 |
| 0.4 | 223.21 | 221.91 | 1.29 |
| 0.5 | 306.97 | 301.91 | 5.06 |
| 0.6 | 522.88 | 517.88 | 5.00 |
| 0.7 | 844.37 | 836.72 | 7.65 |
| 0.8 | 778.61 | 774.17 | 4.44 |
| 0.9 | 534.40 | 532.14 | 2.26 |

obtained from Fig. 10 resulted in negative values of E_a due to the slow degradation rate in response to the increase in the temperature. As shown in Table 6, the E_a values of the statistical analysis resulted from both methods are reliable since the difference between these two methods is < 3% at every α value. Hence, this is proven that both methods adhere to each other and that the gained E_a results are valid.

3.11. Shelf-life prediction

In this section, the shelf-life estimation of LNP12 is predicted at varying temperatures by calculating the E_a of kinetic reaction from TGA data. Termination of LNPs shelf-life occurs when the mass loss reaches 5 wt% ($\alpha = 0.05$) (Othman et al., 2016b). The mass loss ($\alpha = 5\%$) of LNP12 was calculated at different temperatures in nitrogen and air atmospheres according to the shelf-life prediction using Eq. (6) and Eq. (7). Table 7 includes derived data from the calculated E_a value at the 5% conversion (118 kJ mol⁻¹). The value of shelf-life was plotted versus temperature from the second double derivative thermogravimetric (DDTG) curves at 5 °C min⁻¹. Fig. 11a shows the shelf-life prediction (in months) of LNP12 which was performed at the temperature range of 25-100 °C and (b) (in days) conducted at the temperature range of 55-100 °C in a nitrogen atmosphere. According to Fig. 11, the shelf-life decreased with increasing temperature due to the high energy which was used to break the molecular chain in LNP12.

Generally, below 35 °C, LNP12 was stable for a long term which is around 20 months. Hence, the graph in Fig. 11b was plotted to focus on shelf-life prediction in days at a temperature above 45 °C. The shelf-life of LNP12 at temperatures 45 °C and 55 °C was stable for more than 160 days and 40 days, respectively. However, the nano-sized particles started to degrade at a temperature range of 65-100 °C. Overall, the shelf-life of LNP12 drastically decreased to 11 days at 65 °C, 3 days at 75 °C, 25 h at 85 °C, 8 h at 95 °C, and 5 h at 100 °C. Mwangi et al. (2016) reported that the kinetic energy of lignin particles increased with the increase in temperature (20-90 °C) and leads to a rapid collision and random motion of the particles. This creates redistribution of particles towards the interface and thus, increases the chance of coalescing. They also reported that the emulsions were stable at the temperature between 25 and 50 °C. In the present study, although the shelflife decreased with the increase in temperature, the emulsion was prepared at room temperature. In the present study (Fig. 11), the produced LNP was kinetically stable to be applied as an emulsifying agent to emulsions at a temperature below 35 °C. Therefore, storing the emulsion at room temperature for a long period will not damage the product.

Table 7Estimated shelf-life values of NSL12 based on a massloss of 5% at various temperatures.

| β | Ea (kJ mol ⁻¹) | n | A (×10 ¹²) |
|----|----------------------------|------|------------------------|
| 5 | 118.68 | 1.73 | 6.95 |
| 10 | | 1.61 | |
| 15 | | 1.60 | |
| 20 | | 1.38 | |



Fig. 11 Shelf-life prediction of LNP12 (a) in months at the temperature range of 25-100 °C and (b) in days at the temperature range of 55-100 °C in the nitrogen atmosphere.

3.12. Toxicity test

Lignin is a natural phenolic polymer that has not only been proved to be non-toxic but has been also found to have beneficial properties such as being antioxidant (Zhang et al., 2014), while its derivatives also possess antibiotic and anticarcinogenic features (Calvo-Flores and Dobado, 2010). By performing an acute toxicity study, Mendes et al. (2016) reported that lignin is not toxic and showed no significant impact on animals when used in the dosage of 10,000 mg kg⁻¹. However, in this study, lignin was nano-sized and the process of making an emulsifying agent was involving the addition of NaOH and drops of HCl. Hence, it was vital to test the toxicological properties of the selected LNP to determine the safe dosage of the emulsifying agent required to be added to emulsions for human consumption particularly in the food industry.

Since LNP12* formed the most stable suspension and performed the best as an emulsifying agent, it was chosen for the toxicity analysis. As mentioned earlier, the toxicity test was performed using LNP12* *in vitro* (human umbilical vein cell line (ea.hy926) and *in vivo* (rats). The results of the MTT assay showed that the *in vitro* test of LNP12* did not generate any cytotoxic effects on cellular proliferation and morphology of the human endothelial (ea.hy926) cells. Interestingly, LNP12* exhibited a pro-proliferation effect at low concentrations, whereas it showed an anti-proliferation effect at higher concentrations as shown in Fig. 12. Overall, the LNP12* was found to be promoting the proliferation of the endothelial cell at low concentration (<70 µg mL⁻¹). Also, the effect of the sample on viability showed a significantly more proliferation of the cells when compared to the negative control (distilled water).

The graphical representation of the effect of the test sample on cellular viability is demonstrated in Fig. 13a. The IC₅₀ value estimated from Fig. 13b for the sample is 2,928.94 µg (29.28 mg mL⁻¹). Recently, Aćimović et al. (2020) tested the nanosized lignin in the in vitro and found low toxicity at 250 μ g mL⁻¹. The LNP12* was tasted in the concentration range of 1-51,200 μ g mL⁻¹ (0.001-51.2 mg mL⁻¹) found to be non-toxic at a much higher concentration (2,928.94 µg mL ⁻¹) than 250 μ g mL⁻¹. Not to mention, LNP12* is also promoting the proliferation of the endothelial cell at low concentration ($< 70 \ \mu g \ mL^{-1}$). The purpose of the *in vivo* experiment in this study was to assess the acute toxic potential of the test sample when administered to Sprague-Dawley (SD) rats with a single oral dose by gavage followed by physical observations, examination, and gross necropsy. The toxicity of the test sample was compared with negative control (distilled water). According to the test, a single dose of 1000 mg kg^{-1} of the body weight was given to five rats and their mortality was tracked for 48 h. Since no mortality was observed in rats, the dose limit was increased to 2000 mg kg⁻¹ of their body weight. Table 8 classifies the effect of acute administration of LNP12* on neurobehavioral activities of the rats. No clinical signs of toxicity during the tracked period were observed for the rats that were orally fed with the test sample. Also, no mortality occurred during the studied period. The animals were looking healthy and performing their daily normal activity. When the dose of 2000 mg kg⁻¹ of the sample was tested on the rats, no toxicity symptoms, changes in behavior, or mortality were observed. Generally, any substance with LD₅₀ between 1000 and 15,000 mg kg⁻¹ is considered non-toxic (Calvo-Flores and Dobado, 2010). According to the results obtained in the present study, the test sample can be considered non-toxic, as the LD₅₀ for the extract was found to be more than 2000 mg kg⁻¹.

4. Comparative discussion of lignin nanoparticles with previous literature

There are also efforts to substitute emulsifiers from animals and synthetic chemicals to natural plants. The application of lignin as a biologically based surfactant is biocompatible and biodegradable. The emulsifiers are used to stabilize the emulsions that may be affected by various factors such as particle size, particle stability, physiochemical stabilities, and temperature. The TEM images and the LNP size distribution showed that the particles are within the nano range as referring to the present results (<100 nm). In comparison to the previous



Fig. 12 Effect of the test sample on the proliferation of ea.hy926 cells.



Fig. 13 (a) Effect of LNP12* on cellular viability of ea.hy926 cells, (b) Non-linear regression curve of the anti-proliferation effect of the sample to estimate its IC₅₀ value.

| | 7 tillingi 1 | Animai-2 | Animal-3 | Animal-4 | Animal-5 |
|---|---------------------------|---------------------------|---------------|---------------------------|---------------|
| Jnusual posture | ND | ND | ND | ND | ND |
| Jnusual coordination of movement | ND | ND | ND | ND | ND |
| Abnormal gait | ND | ND | ND | ND | ND |
| Headflicking | ND | ND | ND | ND | ND |
| Grooming of the head and neck | Observed at the beginning | ND | ND | Observed at the beginning | ND |
| Compulsive biting or licking | ND | Observed at the beginning | ND | Observed at the beginning | ND |
| self-mutilation | ND | ND | ND | ND | ND |
| Circling | ND | ND | ND | ND | ND |
| Walking backward | ND | ND | ND | ND | ND |
| Convulsions | ND | ND | ND | ND | ND |
| Tremors | ND | ND | ND | ND | ND |
| ncreased levels of lacrimation and/or red-colored tears | ND | ND | ND | ND | ND |
| ncreased levels of salivation | ND | ND | ND | ND | ND |
| Piloerection | ND | ND | ND | ND | ND |
| upillary dilation or constriction. | ND | ND | ND | ND | ND |
| Jnusual respiration (shallow, labored, dyspneic, | ND | ND | Observed at | ND | ND |
| asping, and retching) and/or mouth breathing. | | | the beginning | | |
| Diarrhea. | ND | ND | ND | ND | ND |
| Excessive or diminished urination. | ND | ND | ND | ND | ND |
| Vocalization | ND | ND | ND | ND | Observed at |
| | | | | | the beginning |

Table 8 Effect of acute (single dose) administration of the test sample on neurobehavioral activities.

literature, Nair et al. (2014) produced lignin nanoparticles for four hours at a shear speed of 15,000 rpm, as opposed to the present study where a slower speed of 12,400 rpm was found able to produce a smaller particles size which saves time and energy. The emulsion produced still has strong stability after 30 days even using a small amount of LNP12* (2% w/v). Several researchers found the same way to stabilize emulsion using lignin at various concentrations and particle sizes (Silmore et al., 2016; Li et al., 2016; Bertolo et al., 2019; Shi et al., 2019; Czaikoski et al., 2020) as presented in Table 1. There are not many studies reported on the kinetic thermal stability and the self-life prediction of LNPs. The E_a of lignin that can be associated with collisions and degradation mechanisms was found by Poletto (2017). This current research reveals the shelf-life prediction of LNPs based on Ea results and temperature data. The LNP12 result indicates a very good thermal stability and is projected to have more than 20 months shelf-life if storing at a temperature less than 35 °C.

5. Conclusion

Lignin nanoparticles (LNPs) were prepared as emulsifying agents in the W/O emulsion system. LNPs were produced via homogenization under varied shear speeds (6400 rpm, 8400 rpm, and 12,400 rpm). Although the zeta potential value for SL was the highest, LNP12 (the nanoparticle that was generated at the highest speed of the homogenizer) showed the best size distribution and good zeta potential value which resulted in the most stable emulsion. The CPM image of LNP12** showed the best emulsion by forming small and more uniform droplets. Thus, the LNP12 nanoparticle was chosen for further analyses of kinetic degradation and toxicological studies. The E_a values of LNP12 in the kinetic degradation study were determined via F-W-O and Kissinger methods which were found to be confirming to each other. The LNP12 is found to be better stable and longer shelf-life at a temperature below 35 °C for 20 months. The *in vitro* and *in vivo* toxicological analyses also revealed that LNP12 was a non-toxic substance. Overall, the LNP12* was found to be suitable as an emulsifying agent for industrial applications.

CRediT authorship contribution statement

Asim Ali Yaqoob: Methodology, Visualization, Investigation, Writing - review & editing. Siti Hajar Sekeri: Methodology, Visualization, Investigation. Muhammad Bisyrul Hafi Othman: Conceptualization. Mohamad Nasir Mohamad Ibrahim: Conceptualization, Supervision, Funding acquisition. Zahra Hosseinpour Feizi: Writing - review & editing.

Acknowledgment

The authors would like to express their appreciation to the School of Chemical Sciences, Universiti Sains Malaysia (USM) for the financial support of this study through providing the Universiti Sains Malaysia Grant research grant (304/ PKIMIA/6501153/E128).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Abdelaziz, O.Y., Hulteberg, C.P., 2017. Physicochemical characterisation of technical lignins for their potential valorisation. Waste Biomass Valoriz. 8 (3), 859–869.

- Aćimović, M., Pezo, L., Tešević, V., Čabarkapa, I., Todosijević, M., 2020. QSRR Model for predicting retention indices of Satureja kitaibelii Wierzb. ex Heuff. essential oil composition. Ind. Crops Prod. 154, 112752.
- Ahamed, M.B.K., Aisha, A.F., Nassar, Z.D., Siddiqui, J.M., Ismail, Z., Omari, S., Parish, C., Majid, A.A., 2012. Cat's whiskers tea (Orthosiphon stamineus) extract inhibits growth of colon tumor in nude mice and angiogenesis in endothelial cells via suppressing VEGFR phosphorylation. Nutr. Cancer 64 (1), 89–99.
- Azimvand, J., Didehban, K., Mirshokrai, S.A., 2018. Preparation and characterization of lignin polymeric nanoparticles using the green solvent Ethylene glycol: acid precipitation technology. BioResources 13 (2), 2887–2897.
- Bai, L., Greca, L.G., Xiang, W., Lehtonen, J., Huan, S., Nugroho, R. W.N., Tardy, B.L., Rojas, O.J., 2018. Adsorption and assembly of cellulosic and lignin colloids at oil/water interfaces. Langmuir 35 (3), 571–588.
- Beisl, S., Friedl, A., Miltner, A., 2017. Lignin from micro-to nanosize: applications. Int. J. Mol. Sci. 18 (11), 2367.
- Bertolo, M.R., de Paiva, L.B.B., Nascimento, V.M., Gandin, C.A., Neto, M.O., Driemeier, C.E., Rabelo, S.C., 2019. Lignins from sugarcane bagasse: Renewable source of nanoparticles as Pickering emulsions stabilizers for bioactive compounds encapsulation. Ind. Crops Prod. 140, 111591.
- Bove, D., Moliner, C., Curti, M., Baratieri, M., Bosio, B., Rovero, G., Arato, E., 2019. Preliminary tests for the thermo-chemical conversion of biomass in a spouted bed pilot plant. Canadian J. Chem. Eng. 97 (1), 59–66.
- Calvo-Flores, F.G., Dobado, J.A., 2010. Lignin as renewable raw material. ChemSusChem 3 (11), 1227–1235.
- Chen, W.-H., Kuo, P.-C., 2011. Isothermal torrefaction kinetics of hemicellulose, cellulose, lignin and xylan using thermogravimetric analysis. Energy 36 (11), 6451–6460.
- Chen, X., Che, Q., Li, S., Liu, Z., Yang, H., Chen, Y., Wang, X., Shao, J., Chen, H., 2019. Recent developments in lignocellulosic biomass catalytic fast pyrolysis: Strategies for the optimization of bio-oil quality and yield. Fuel Process. Technol. 196, 106180.
- Cortés, A.M., Bridgwater, A., 2015. Kinetic study of the pyrolysis of miscanthus and its acid hydrolysis residue by thermogravimetric analysis. Fuel Process. Technol. 138, 184–193.
- Czaikoski, A., Gomes, A., Kaufmann, K.C., Liszbinski, R.B., de Jesus, M.B., da Cunha, R.L., 2020. Lignin derivatives stabilizing oil-in-water emulsions: Technological aspects, interfacial rheology and cytotoxicity. Ind. Crops Prod. 154, 112762.
- Delgado, N., Ysambertt, F., Chávez, G., Bravo, B., García, D.E., Santos, J., 2019. Valorization of Kraft Lignin of Different Molecular Weights as Surfactant Agent for the Oil Industry. Waste Biomass Valoriz. 10 (11), 3383–3395.
- Figueiredo, P., Lintinen, K., Kiriazis, A., Hynninen, V., Liu, Z., Bauleth-Ramos, T., Rahikkala, A., Correia, A., Kohout, T., Sarmento, B., 2017. In vitro evaluation of biodegradable ligninbased nanoparticles for drug delivery and enhanced antiproliferation effect in cancer cells. Biomaterials 121, 97–108.
- Gupta, A., Eral, H.B., Hatton, T.A., Doyle, P.S., 2016. Nanoemulsions: formation, properties and applications. Soft Matter 12 (11), 2826–2841.
- Gupta, C., Washburn, N.R., 2014. Polymer-grafted lignin surfactants prepared via reversible addition–fragmentation chain-transfer polymerization. Langmuir 30 (31), 9303–9312.
- Hattalli, S., Benaboura, A., Ham-Pichavant, F., Nourmamode, A., Castellan, A., 2002. Adding value to Alfa grass (Stipa tenacissima L.) soda lignin as phenolic resins 1. Lignin characterization. Polym. Degrad. Stab. 76 (2), 259–264.
- Henn, A., Mattinen, M.-L., 2019. Chemo-enzymatically prepared lignin nanoparticles for value-added applications. World J. Microbiol. Biotechnol. 35 (8), 1–9.
- Hussin, M.H., Samad, N.A., Latif, N.H., Rozuli, N.A., Yusoff, S.B., Gambier, F., Brosse, N., 2018. Production of oil palm (Elaeis

guineensis) fronds lignin-derived non-toxic aldehyde for ecofriendly wood adhesive. Int. J. Biol. Macromol. 113, 1266–1272.

- Ibrahim, M.M., Chuah, S., Rosli, W.W., 2004. Characterization of lignin precipitated from the soda black liquor of oil palm empty fruit bunch fibers by various mineral acids. ASEAN J. Sci. Technol. Develop. 21 (1), 57–67.
- Ibrahim, M.N.M., Zakaria, N., Sipaut, C.S., Sulaiman, O., Hashim, R., 2011. Chemical and thermal properties of lignins from oil palm biomass as a substitute for phenol in a phenol formaldehyde resin production. Carbohydr. Polym. 86 (1), 112–119.
- Jiang, G., Nowakowski, D.J., Bridgwater, A.V., 2010. A systematic study of the kinetics of lignin pyrolysis. Thermochim. Acta 498 (1– 2), 61–66.
- Lee, R.A., Bédard, C., Berberi, V., Beauchet, R., Lavoie, J.-M., 2013. UV–Vis as quantification tool for solubilized lignin following a single-shot steam process. Bioresour. Technol. 144, 658–663.
- Li, S., Willoughby, J.A., Rojas, O.J., 2016. Oil-in-Water Emulsions Stabilized by Carboxymethylated Lignins: Properties and Energy Prospects. ChemSusChem 9 (17), 2460–2469.
- Lievonen, M., Valle-Delgado, J.J., Mattinen, M.-L., Hult, E.-L., Lintinen, K., Kostiainen, M.A., Paananen, A., Szilvay, G.R., Setälä, H., Österberg, M., 2016. A simple process for lignin nanoparticle preparation. Green Chem. 18 (5), 1416–1422.
- Mani, T., Murugan, P., Mahinpey, N., 2009. Determination of distributed activation energy model kinetic parameters using simulated annealing optimization method for nonisothermal pyrolysis of lignin. Ind. Eng. Chem. Res. 48 (3), 1464–1467.
- Matsakas, L., Karnaouri, A., Cwirzen, A., Rova, U., Christakopoulos, P., 2018. Formation of lignin nanoparticles by combining organosolv pretreatment of birch biomass and homogenization processes. Molecules 23 (7), 1822.
- Mendes, A.E., Tonin, F.S., Fernandez-Llimos, F., 2016. Analysis of ten years of publishing in Pharmacy Practice. Pharmacy Practice (Granada) 14 (4).
- Mwangi, W.W., Ho, K.-W., Tey, B.-T., Chan, E.-S., 2016. Effects of environmental factors on the physical stability of pickeringemulsions stabilized by chitosan particles. Food Hydrocolloids 60, 543–550.
- Nair, S.S., Sharma, S., Pu, Y., Sun, Q., Pan, S., Zhu, J., Deng, Y., Ragauskas, A.J., 2014. High shear homogenization of lignin to nanolignin and thermal stability of Nanolignin-Polyvinyl alcohol blends. ChemSusChem 7 (12), 3513–3520.
- Nypelö, T.E., Carrillo, C.A., Rojas, O.J., 2015. Lignin supracolloids synthesized from (W/O) microemulsions: use in the interfacial stabilization of Pickering systems and organic carriers for silver metal. Soft Matter 11 (10), 2046–2054.
- Othman, M.B.H., Akil, H.M., Khan, A., Ahmad, Z., Rasib, S.Z.M., 2016a. Kinetic and Solid State Degradation of Chitosan-Methacrylic acid-N, N-methylenebisacrylamide (Cs-MAA-MBA) Dual-responsive Hydrogels.
- Othman, M.B.H., Akil, H.M., Rasib, S.Z.M., Khan, A., Ahmad, Z., 2015. Thermal properties and kinetic investigation of chitosan-PMAA based dual-responsive hydrogels. Ind. Crops Prod. 66, 178– 187.
- Othman, M.B.H., Khan, A., Ahmad, Z., Zakaria, M.R., Ullah, F., Akil, H.M., 2016b. Kinetic investigation and lifetime prediction of Cs-NIPAM-MBA-based thermo-responsive hydrogels. Carbohydr. Polym. 136, 1182–1193.
- Pasangulapati, V., Ramachandriya, K.D., Kumar, A., Wilkins, M.R., Jones, C.L., Huhnke, R.L., 2012. Effects of cellulose, hemicellulose and lignin on thermochemical conversion characteristics of the selected biomass. Bioresour. Technol. 114, 663–669.
- Poletto, M., 2017. Assessment of the thermal behavior of lignins from softwood and hardwood species. Maderas. Ciencia y tecnología 19 (1), 63–74.
- Qian, Y., Zhang, Q., Qiu, X., Zhu, S., 2014. CO 2-responsive diethylaminoethyl-modified lignin nanoparticles and their applica-

tion as surfactants for CO 2/N 2-switchable Pickering emulsions. Green Chem. 16 (12), 4963–4968.

- Rashid, T., Gnanasundaram, N., Appusamy, A., Kait, C.F., Thanabalan, M., 2018. Enhanced lignin extraction from different species of oil palm biomass: Kinetics and optimization of extraction conditions. Ind. Crops Prod. 116, 122–136.
- Rousseau, D., 2000. Fat crystals and emulsion stability—a review. Food Res. Int. 33 (1), 3–14.
- Salerno, A., Bolzinger, M.-A., Rolland, P., Chevalier, Y., Josse, D., Briançon, S., 2016. Pickering emulsions for skin decontamination. Toxicol. In Vitro 34, 45–54.
- Sapei, L., Damayanti, O.P., 2017. Kinetics of oil-in-water emulsion stabilization using lecithin and biosilica. ASEAN J. Chem. Eng. 17 (1), 1–7.
- Sekeri, S.H., Ibrahim, M.N.M., Umar, K., Yaqoob, A.A., Azmi, M. N., Hussin, M.H., Othman, M.B.H., Malik, M.F.I.A., 2020. Preparation and characterization of nanosized lignin from oil palm (Elaeis guineensis) biomass as a novel emulsifying agent. Int. J. Biol. Macromol. 164, 3114–3124.
- Shi, Z., Ouyang, Y., Qiu, R., Hu, S., Zhang, Y., Chen, M., Wang, P., 2019. Bioinspired superhydrophobic and oil-infused nanostructured surface for Cu corrosion inhibition: a comparison study. Prog. Org. Coat. 131, 49–59.
- Silmore, K.S., Gupta, C., Washburn, N.R., 2016. Tunable Pickering emulsions with polymer-grafted lignin nanoparticles (PGLNs). J. Colloid Interface Sci. 466, 91–100.
- Singh, S.K., Dhepe, P.L., 2016. Isolation of lignin by organosolv process from different varieties of rice husk: understanding their physical and chemical properties. Bioresour. Technol. 221, 310– 317.
- Sipponen, M.H., Smyth, M., Leskinen, T., Johansson, L.-S., Österberg, M., 2017. All-lignin approach to prepare cationic colloidal lignin particles: stabilization of durable Pickering emulsions. Green Chem. 19 (24), 5831–5840.
- Skreiberg, A., Skreiberg, Ø., Sandquist, J., Sørum, L., 2011. TGA and macro-TGA characterisation of biomass fuels and fuel mixtures. Fuel 90 (6), 2182–2197.
- Sun, R., Lu, Q., Sun, X., 2001. Physico-chemical and thermal characterization of lignins from Caligonum monogoliacum and Tamarix spp. Polym. Degrad. Stab. 72 (2), 229–238.

- Sun, R., Tomkinson, J., Bolton, J., 1999. Effects of precipitation pH on the physico-chemical properties of the lignins isolated from the black liquor of oil palm empty fruit bunch fibre pulping. Polym. Degrad. Stab. 63 (2), 195–200.
- Tian, D., Hu, J., Bao, J., Chandra, R.P., Saddler, J.N., Lu, C., 2017. Lignin valorization: Lignin nanoparticles as high-value bio-additive for multifunctional nanocomposites. Biotechnol. Biofuels 10 (1), 1– 11.
- Tong, Y., Wang, Y., Yang, M., Yang, J., Chen, L., Chu, X., Gao, C., Jin, Q., Gong, W., Gao, C., 2018. Systematic development of selfnanoemulsifying liquisolid tablets to improve the dissolution and oral bioavailability of an oily drug, vitamin K1. Pharmaceutics 10 (3), 96.
- Ur Rahman, O., Shi, S., Ding, J., Wang, D., Ahmad, S., Yu, H., 2018. Lignin nanoparticles: synthesis, characterization and corrosion protection performance. New J. Chem. 42 (5), 3415–3425.
- Wei, Z., Yang, Y., Yang, R., Wang, C., 2012. Alkaline lignin extracted from furfural residues for pH-responsive Pickering emulsions and their recyclable polymerization. Green Chem. 14, 3230–3236.
- Wellen, R.M., Canedo, E.L., 2014. On the Kissinger equation and the estimate of activation energies for non-isothermal cold crystallization of PET. Polym. Test. 40, 33–38.
- Xin, X., Zhang, H., Xu, G., Tan, Y., Zhang, J., Lv, X., 2013. Influence of CTAB and SDS on the properties of oil-in-water nano-emulsion with paraffin and span 20/Tween 20. Colloids Surf., A 418, 60–67.
- Yaqoob, A.A., Ahmad, H., Parveen, T., Ahmad, A., Oves, M., Ismail, I.M., Qari, H.A., Umar, K., Mohamad Ibrahim, M.N., 2020. Recent advances in metal decorated nanomaterials and their various biological applications: a review. Front. Chem. 8, 341.
- Zhang, J., Chen, T., Wu, J., Wu, J., 2014. A novel Gaussian-DAEMreaction model for the pyrolysis of cellulose, hemicellulose and lignin. RSC Adv. 4 (34), 17513–17520.
- Zhang, Y., Yang, M., Portney, N.G., Cui, D., Budak, G., Ozbay, E., Ozkan, M., Ozkan, C.S., 2008. Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells. Biomed. Microdev. 10 (2), 321–328.
- Zhou, H., Long, Y., Meng, A., Chen, S., Li, Q., Zhang, Y., 2015. A novel method for kinetics analysis of pyrolysis of hemicellulose, cellulose, and lignin in TGA and macro-TGA. RSC Adv. 5 (34), 26509–26516.