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Pristine and Iodo-Lignin Nanoparticles Prepared via Nanoprecipitation for Antifungal Applications

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

In this study, lignin and iodo-lignin nanoparticles were prepared by nanoprecipitation method. Lignin nanoparticles (LNPs) demonstrated a decrease in particle size from 200 nm to 20 nm with an increased volume of water from 10 mL to 50 mL. Meanwhile, the obtained lignin nanoparticles are functionalised with iodine by laccase catalysed iodination. Iodo-lignin nanoparticles (I-LNPs) shows decreased nanosized from 350 nm to 40 nm with decreased volume of water (50 mL to 10 mL) after observed under SEM and TEM analysis. Further, antifungal activity was evaluated on LNPs and I-LNPs at different particles size (20 nm to 350 nm) and different concentrations (3 mM to 12 mM) against *Fusarium equiseti*, *Cunninghamella* sp., *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma piluliferum* and *Penicillium chermesinum*. The maximum antifungal activity was achieved by decreasing nanosized and increasing the concentration of LNPs and I-LNPs. Mycelium inhibition of I-LNPs were greatly decreased about 65% to 98 % with decreasing nanosized and increasing concentration against the tested fungi. The best antifungal results were achieved using I-LNPs size at 40 nm combined with 12 mM concentration. Meanwhile, LNPs demonstrated lower inhibition about 20% to 75%. Similarly, the best result for LNPs was achieved using the smallest nanosized at 20 nm combined with 12 mM concentration. The potential application of LNPs and I-LNPs were further tested on bamboo by impregnation of nanoparticles via laccase catalysed reaction. The molecular results revealed the isolated fungi as *Cunninghamella* sp., *Pleosporales* sp. and *P. sumatrense.* Then, these fungi were tested against LNPs and I-LNPs. Interestingly, I-LNPs shows promising results about 80% to 90% of mould resistance against *Cunninghamella* sp., *Pleosporales* sp., and *P. sumatrense.* Further decay resistance test of I-LNPs treated bamboo shows excellent results with less than 5% of mass loss. After leaching, I-LNPs shows good decay resistance with less than 8 % of mass loss after 90 days of incubation. This work has revealed that I-LNPs and LNPs can be a potential candidate as antifungal agents against plant pathogenic fungi.

Keywords: Lignin nanoparticles, iodo-lignin nanoparticles, antifungal activity, mould resistance, decay resistance

Nanopartikel Asli dan Iodo-lignin yang Disediakan melalui Pemendakan untuk Aplikasi Antikulat

ABSTRAK

Dalam kajian ini, nanopartikel lignin dan iodo-lignin telah disediakan dengan kaedah pemendakan nano. Nanopartikel lignin (NPL) menunjukkan penurunan saiz zarah daripada 200 nm kepada 20 nm dengan peningkatan isipadu air daripada 10mL hingga 50 mL. Sementara itu, nanopartikel lignin yang diperoleh difungsikan dengan iodin melalui pengionan bermangkin lakase. Nanopartikel iodo-lignin (NPIL) menunjukkan saiz zarah yang berkurangan daripada 300 nm kepada 40 nm dengan penurunan isipadu air (50 mL hingga 10 mL) selepas diperhatikan di bawah analisis SEM dan TEM. Selanjutnya, aktiviti antikulat dinilai pada NPL dan NPIL pada saiz zarah yang berbeza dan kepekatan berbeza (3 mM hingga 12 mM) terhadap Fusarium equiseti, Cunninghamella sp., Aspergillus niger, Aspergillus flavus, Trichoderma piluliferum dan Penicillium chermesinum. Aktiviti antikulat maksimum dicapai dengan mengurangkan saiz nano dan meningkatkan kepekatan NPL dan NPIL. Perencatan miselium NPIL telah berkurangan dengan banyak iaitu kira-kira 65% hingga 98% dengan pengurangan saiz nano dan peningkatan kepekatan terhadap kulat yang diuji. Keputusan antikulat terbaik dicapai menggunakan saiz NPIL pada 40 nm digabungkan dengan kepekatan 12 mM. Sementara itu, NPL menunjukkan perencatan yang lebih rendah kira-kira 20% hingga 75%. Begitu juga, keputusan terbaik untuk NPL dicapai menggunakan saiz NPL terkecil pada 20 nm digabungkan dengan kepekatan 12 mM. Potensi aplikasi NPL dan NPIL telah diuji selanjutnya pada buluh dengan impregnasi nanopartikel melalui tindak balas pemangkin lakase. Kulat yang dikenal pasti secara molekular daripada buluh telah dikenal pasti sebagai Cunninghamella sp. dan Pleosporales sp. dan P.

sumatrense. Kemudian, kulat ini diuji terhadap NPL dan NPIL. Menariknya, NPIL menunjukkan hasil yang menjanjikan dengan kira-kira 80% hingga 90% rintangan kulat terhadap Cunninghamella sp., Pleosporales sp. dan P. sumatrense. Ujian rintangan pereputan selanjutnya menunjukkan keputusan yang sangat baik dengan kurang daripada 5% kehilangan jisim untuk NPIL yang tidak larut lesap. Selepas larut lesap, NPIL menunjukkan rintangan pereputan yang baik dengan kurang daripada 8% kehilangan jisim selepas 90 hari pengeraman. Kerja ini telah mendedahkan bahawa NPIL dan NPL boleh menjadi calon berpotensi sebagai agen antikulat terhadap kulat tumbuhan yang patogenik.

Kata kunci: *Nanopartikel lignin, nanopartikel iodo-lignin, aktiviti antikulat, rintangan kulat, rintangan pereputan*

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LIST OF ABBREVIATIONS

ABTS	2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ASTM	American Society for Testing and Materials Standard
AWPA	American Wood Protection Association Standard
CCA	Copper Chrome Arsenic
EDX	Electron Dispersive X-Ray
FT-IR	Fourier Transformed Infra Ray
I-LNPs	Iodo-Lignin Nanoparticles
KI	Potassium Iodide
LNPs	Lignin Nanoparticles
MG	Mean Growth
mM	Millimolar
nm	Nanometre
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscopy
UV	Ultraviolet
UV-vis	Ultraviolet Visible Spectrophotometer

CHAPTER 1

INTRODUCTION

1.1 Study Background

Lignin is the most abundant aromatic polymer and the second most abundant biopolymer after cellulose. It is a complex biopolymer composed of different amounts of monolignols, namely p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Ralph et al., 2019; Schneider et al., 2021). Lignin can be used in industry because it is low-cost, non-toxic, biodegradable, and biocompatible.

Recently, researchers have gained interest on lignin-based nanoparticles due to its improved antifungal activity (Gordobil et al., 2018; Morales et al., 2022). Various functional groups of thiols, phenols and hydroxyl groups make lignin an ideal precursor for functionalisation with various compounds (Cui et al., 2021). Therefore, lignin-based nanoparticles have been applied in various applications such as antioxidant agents (Trevisan & Rezende, 2020), drug delivery carriers (Yiamsawas et al., 2017) and antibacterial agents (Morena et al., 2022). Also, lignin has been reported to exhibit antifungal activity against plant pathogenic fungi. For instance, Gordobil et al. (2018) reported that alkali lignin has weak antifungal activity against pathogenic fungi such as *Aspergillus niger* and *Aspergillus flavus.* Therefore, lignin in combination with another antifungal agent could be used as a promising strategy to enhance its antifungal properties (Jose et al., 2020; Tran et al., 2023).

Meanwhile, Cuellar-Rufino et al. (2022) reported that inorganic compound such as iodine is an effective antiseptic and disinfectant against fungi, viruses, and bacteria. It was reported that iodine and iodocompounds exhibit good antifungal activity at millimolar concentrations against plant pathogenic fungi such as *Fusarium*, *Cunninghamella*, *Aspergillus*, and *Penicillium* species (Wei et al., 2022; Wójcik, 2023). However, iodine has several limitations, which include skin discoloration, instability, and irritation. Utilising iodine-binding and releasing polymers (iodophores) can overcome these limitations. For instance, polymer-based iodine such as polyvinylpyrrolidone is a promising way to overcome the limitation of iodine as antiseptic (Pérez-Pazos et al., 2023; Dattilo et al., 2023). Therefore, a combination of lignin nanoparticles and iodine is a great strategy to enhance the antifungal activity of lignin.

The functionalisation of iodine with lignin can be done by using laccase mediated reaction. Laccase is a multicopper oxidase, which is responsible for the oxidation of a wide variety of aromatic and non-aromatic substances (Zerva et al., 2019; Nikolaivits et al., 2021). In the case of lignin, laccase can cleave bond; the bonds between C_1 and C_2 carbons, known as C_α–C_β cleavage, and between C_α and the aryl group, which is known as alkyl–aryl cleavage (Kawai et al., 1999; Zheng et al., 2019). Subsequently, the laccase mediated reaction will bind the aromatic ring of lignin with other inorganic compound such as iodine. The electrons that are produced by the oxidation of the substrate are transferred to molecular oxygen, which is then reduced to potassium hydroxide (KOH) (Sdahl et al., 2019). It is suggested that the utilisation of redox mediator such as 2,2’-azino-bis(3-ethylbenzalthiazoline-6-sulphonic acid) (ABTS) can significantly increases the rates at which iodide oxidation occurs (Li et al., 2019).

The potential of laccase mediated system has further been explored in impregnation of phenolic compound on bamboo. According to Prosper et al. (2016), laccase mediated system could bind iodine on bamboo surface to impart its antifungal resistance. Meanwhile, Wang et al. (2020) demonstrated that laccase was applied to incorporate thymol on the bamboo surface with high leaching resistance. Therefore, a combination of iodine and lignin nanoparticles could work perfectly well against fungi.

1.2 Problems Statement

Nowadays, plant diseases such as crown rot, leaf blast and scab are serious diseases caused by pathogenic fungi (Jaiswal et al., 2020; Sethy et al., 2020; Baboo et al., 2021; Mondal et al., 2022). Controlling fungal infections via chemical fungicides are not only costly but also can cause environmental problems. For instance, chemical fungicides such as captan, dithiocarbamates and pentachlorophenol are toxic to human health (Vogel et al., 2019; Ranjan et al., 2021; Ajiboye et al., 2022). In addition, the leaching of these chemicals into soils can harm the environment (Yuan et al., 2019; Fanjul-Bolado et al., 2020).

Recently, researchers have gained interest in the application of biopolymer-based nanoparticles derived from lignin as potential antifungal agent. However, lignin is a weak antifungal agent (Gordobil et al., 2018; Jose et al., 2020). Therefore, a combination of lignin with another antifungal agent can enhance its antifungal properties. According to U.S Food and Drug Administration (FDA), iodine can be safely consumed at microgram concentration (Pehrsson et al., 2022). However, iodine is not effective against fungi at microgram/mL or micromolar concentration (Pérez-Pazos et al., 2023). Also, high concentration of iodine as a fungicide is toxic to human and environment. Therefore, functionalisation of lignin-based nanoparticles is essential to improve its antifungal activity.

Apart from that, culm blight, culm rot and witches' broom are serious bamboo diseases caused by rot-fungi (Jiang et al., 2018; Ravi et al., 2022; Lin et al., 2023). Generally, chemical preservatives are costly and toxic to human and environment. For instance, inorganic compounds such as boron can cause serious problems to human health and environment (Gauss et al., 2020). Besides that, chemical preservatives such as copper chrome arsenic (CCA) and chlorothalonil have carcinogenic properties which can cause serious problems to human health (Kilpi-Koski et al., 2020; Morais et al., 2021). To address these issues, lignin-based nanoparticles are a wise option to be applied as antifungal agents due to its low cost, non-toxic, biodegradable, and biocompatible features.

1.3 Objectives

The specific objectives of this study are:

i. To synthesise lignin nanoparticles (LNPs) and iodo-lignin nanoparticles (I-LNPs) through nanoprecipitation method in aqueous medium.

ii. To characterise the surface morphology, chemical and antifungal properties of lignin nanoparticles and iodo-lignin nanoparticles.

iii. To evaluate the potential application of lignin-based nanoparticles for bamboo protection.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Lignin

Lignin is a complex and irregular biopolymer found in plant secondary cell walls. This aromatic compound consists of monolignol units, the lignin building blocks such as p-hydroxyphenyl, guaiacyl, and syringyl (Schubert et al., 2021; Afifi et al., 2022). According to Weng et al. (2021), this monolignol units are linked via various types of linkages, primarily ether bonds (such as aryl) and carbon-carbon bonds (Figure 2.1). Lignin can be found in vascular plants' middle lamella and secondary cell walls. The cellulose fibrils are encased in lignin and hemicellulose, which give the plant mechanical support and facilitate water conduction (Vanholme et al., 2019). In addition to its structural role, the polyphenolic composition of lignin and its hydrophobic nature ensure the resistance of plants to biological and chemical degradation and contribute to their defence against pathogens (Melro et al., 2021; Shu et al., 2021).

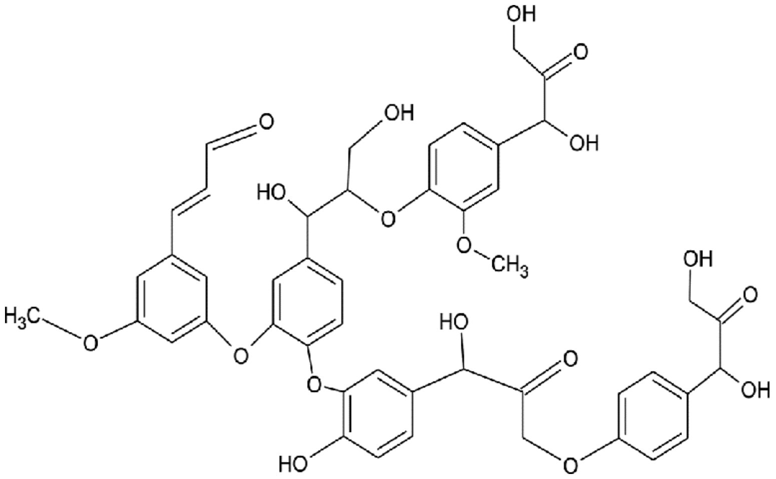

Figure 2.1: Structure of Lignin

The main extraction process of alkali lignin in industry is kraft process (Demuner et al., 2019; Zhao et al., 2019). Kraft process commonly occurs in alkaline media, where lignin is broken into low molecular weight fragments, resulting changes in its physical-chemical properties (Jiang et al., 2019; Acciardo et al., 2022). In kraft procedure, sodium hydroxide and sodium sulphide are added to break the lignin-carbohydrate linkage and give rise to chemically resistant isolated cellulose. This process occurs at a temperature of 180 °C for 2 hours until dark liquor produced, comprising a mixture of inorganic pulping chemicals, hemicellulose, and lignin (Crestini et al., 2017; Budnyak et al., 2020).

The production of alkali lignin by kraft process has a high purity because it contains less derivatives such as sulphur, carbohydrate residues and inorganic impurities (Zhao et al., 2020; Lotfy & Basta, 2022). It also has high antifungal activity compared to organosolv and soda lignin (Morales et al., 2022; Ullah et al., 2022). Due to these properties, alkali lignin is a good choice as raw materials for antifungal study.

2.2 Synthesis of Lignin Nanoparticles

There are several approaches reported in the synthesis of lignin nanoparticles. Several approaches that are widely studied are mechanical and nanoprecipitation methods (Manisekaran et al., 2022; Matsakas et al., 2020; Richter et al., 2016).

2.2.1 Mechanical Method

The high-pressure homogenisation and stirring is a mechanical method usually used in many technical applications due to high efficiency and ease of scaling up. This method could promote the conversion of microsize lignin into nanosize lignin. For instance, Matsakas et al. (2020) reported that the synthesis of lignin nanoparticles by homogenisation could produce lignin nanoparticles with sizes of 10 nm to 100 nm after 4 hours of mechanical shearing. Similarly, Chang et al. (2019) reported a decreased of nanosize from 200 nm to 100 nm by increasing the stirring rate. In addition, a combination of homogenisation and stirring rate could effectively reduce the particle size. For instance, Mo et al. (2021) reported a decreased in particle size from 300 nm to 100 nm after homogenised and subsequently stirred for 1 hour. Similarly, Tian et al. (2021) demonstrate a decreased of particle size from 1 µm to 100 nm after homogenisation and stirring at 150 rpm for 1 hour.

In addition, the chemical composition and molecular weight distribution of lignin nanoparticles were not affected after homogenised and stirred (Nair et al., 2014; Wijaya et al., 2021). To conclude, a combination of stirring and homogenisation could effectively decrease the size of lignin nanoparticles.

2.2.2 Nanoprecipitation by Solvent Shifting Method

Solvent shifting is regarded as one of the most promising nanoparticle synthesis methods for leveraging green chemistry in industrial applications due to its low cost and simplicity. Typically, the solvent shifting system to synthesise lignin nanoparticles consists of a mixture of organic solvent and water (Cailotto et al., 2020; Österberg et al., 2020; Ma et al., 2023).

In solvent shifting, lignin was first dissolved into water-miscible solvents such as acetone. The resulting lignin solution was shifted with an excess amount of anti-solvent such as water, leading to the formation of lignin nanoparticles via self-assembly (Richter et al., 2016; Leskinen et al., 2017). According to Ago et al. (2017), the supramolecular interactions between aromatic lignin structures and internal H-bonding led to formation of spherical lignin nanoparticles via self-assembly (Figure 2.1).

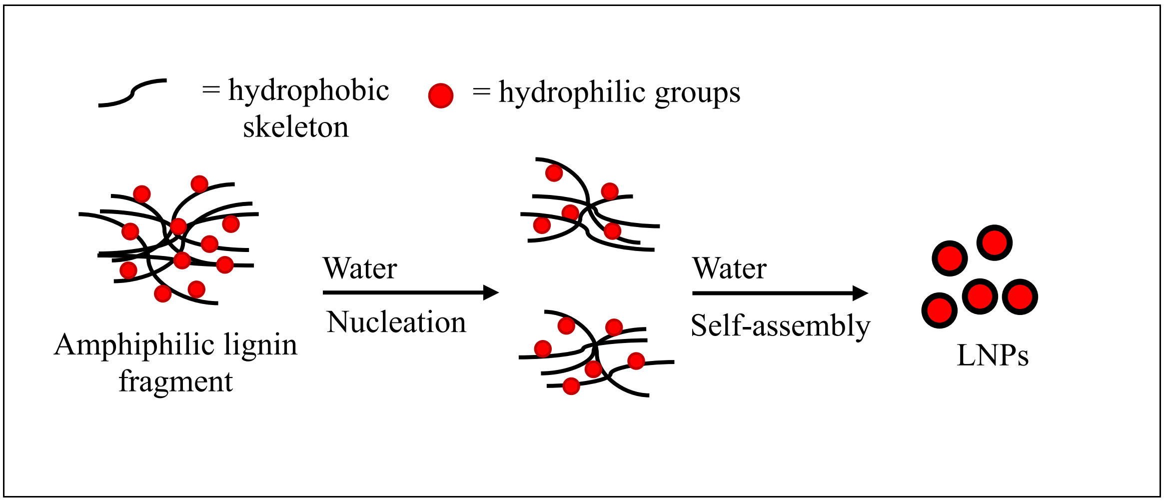

Figure 2.2: The Formation of Lignin Nanoparticles via Self-Assembly (Ago et al., 2017)

In solvent shifting, the size and stability of nanoparticles are affected by the volume of antisolvent, such as water. For instance, Manisekaran et al. (2022) reported a decreased in particles sizes of lignin-based nanoparticles when increased water-to-solvent ratio. The prepared nanoparticles were decreased from 200 nm to 50 nm with increased volume of water from 4 mL to 20 mL. Also, the prepared LNPs by solvent shifting demonstrates superior colloidal stability in water dispersion. Similarly, Lee et al. (2021) prepared spherical LNPs ranged from 100 nm to 20 nm with increasing volume of water in solvent THF. Meanwhile, Richter et al. (2016) prepared LNPs in acetone-water system. They concluded that the prepared LNPs demonstrate a decreased in particle sizes from 300 nm to 50 nm after increasing volume of water from 10 mL to 50 mL of ultrapure water.

In addition, a combination method of solvent shifting, and mechanical stirring could be used to effectively decrease the size of lignin-based nanoparticles. For instance, Lievonen