Lignins and their potential for use as biopolymers in pharmaceutical engineering

Les lignines et leur potentiel d'utilisation en tant que biopolymères dans le domaine du génie pharmaceutique

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Abstract : Nature offers a diverse set of renewable biopolymers, among them the variety of natural polyphenols, whose structural diversity provides opportunities for further manipulation like the generation of chemicals and novel functional materials. These land-based renewable biomaterials exhibit also a wide range of heterogeneous intrinsic reactivities and activities that render them ideal starting oligomeric and polymeric materials for the generation of multifunctional supramolecular structures as well as the development of high value-added materials.

Lignins in particular display a variety of common physico-chemical features that can be of enormous interest concerning their eventual use in the pharmaceutical area. These natural polyphenols possess some interesting pharmaceutical properties such as antioxidant, anti-inflammatory, antibacterial and eventually antiviral activities. Based on their structural features, i.e., oligomeric and/or polymeric structures of monolignol C9-building blocks, lignins can be exploited in the pharmaceutical sector as material for generation of matrices and carriers for drug delivery. The present work attempts a review of the state of the art in lignin use in pharmaceutical applications.

Key words : biopolymers, lignins, microcapsules, nanoparticles, renewable resources

Résumé

La nature offre un ensemble diversifié de biopolymères renouvelables, parmi lesquels la variété de polyphénols naturels, dont la diversité structurelle offre des possibilités de manipulation ultérieure comme la génération de produits chimiques et de nouveaux matériaux fonctionnels. Ces biomatériaux renouvelables présentent également un large éventail de réactivités et d'activités intrinsèques hétérogènes qui font d'eux des matériaux qualifiés pour la génération de structures supramoléculaires multifonctionnelles et pour le développement de matériaux à haute valeur ajoutée. Les lignines en particulier présentent une variété de caractéristiques physico-chimiques communes qui peuvent être d'un intérêt majeur quant à leur utilisation éventuelle dans l'ingénierie pharmaceutique. En effet, ces polyphénols naturels possèdent des propriétés pharmaceutiques intéressantes telles que des activités antioxydantes, anti-inflammatoires, antibactériennes et éventuellement antivirales. En outre, en fonction de leurs caractéristiques chimiques, c'est-dire des oligomères et / ou des polymères de types monolignol C9. Les lignines peuvent donc être exploitées dans le secteur pharmaceutique principalement comme matériau pour la génération de matrices et de véhicules pour l'administration de médicaments. La présente étude est une vue panoramique de l'état de l'art dans l'utilisation de la lignine dans les applications pharmaceutiques.

Mots-clés : Biopolymères, Lignines, Microcapsules, Nanoparticules, Ressources renouvelables

Introduction

From all the renewable components present in land-based and aqueous biomass, natural polyphenols, namely lignin, lignans and tannins, represent important structural materials in the support tissues of vascular plants and/or valuable products of secondary plant metabolism that have a fundamental role in different stages of plant life.

Lignins in particular are structural secondary metabolites mainly for supporting plant growth in terms of giving stability, and by protecting them additionally against attacking organisms.

In average, up to one third of lignocellulosic biomass is comprised of polyphenolic oligomers and polymers, which are for the most part lignins.(1–4) The different functions of lignin in the plant lead to varying distributions across the different parts of the plant, *i.e.*, stems, branching points, branches and leaves, and between the different plant cell walls.(5,6) The majority of the total

amount of lignin present in the plant, 75 – 85 %, is located in the secondary wall, due to its considerably large volume. Lignin abundancy is different for every plant species, ranging from ca. 20 % in hardwoods, ca. 28 % in softwoods and herbaceous angiosperms, to ca. 15 % in monocots.(1,5,7,8)

Lignins are synthesized in vascular plants by radical polymerization of 4-hydroxyphenylpropane units, leading to structurally complex polyphenolic structures. An even larger variety and diversity are caused by the fact that different wood species, in combination with various extraction processes, result in different structural characteristics of available isolated lignin species.(3,9-11)

Lignins display intrinsic features that render them ideal ingredients for applications in the biomedical sector.(6,12) They possess: i) different functional groups along their phenolether backbone that render them predisposed for functionalization with, for example, surface-active groups; ii) different functional groups along their phenol ether backbone that confer to them an amphiphilic character; iii) phenolic groups that allow them to undergo π-anion(13) and π-hydrogen/hydroxyl(14) interactions; iv) phenolic groups susceptible to oxidation, rendering lignins natural antioxidants.

The complexity and heterogeneity of chemical and structural features in lignin make it difficult to arrive at detailed structural characterization and the slow adaption of new structural insights by the wider community, which make their potential somewhat underexploited. Nevertheless, the complex and heterogenous chemical features have been increasingly exploited in the recent past in form of lignin-based or lignin-containing applications in large scale and specialized materials sector,(15–20) in home care applications like laundry and surface cleaning products, in functional cosmetics and for pharmaceuticals, *e.g.*, in terms of microcarriers for drug delivery approaches(21–24) and biomedical uses.(10,15–20,23,25–28) In addition, a suitable valorisation of lignin waste streams from pulp and paper and modern biorefinery processes may be a crucial step for the development of a circular sustainable economy, nowadays.

The highly available diverse chemical features in isolated lignins can be exploited for the generation of multifunctional supra-molecular structures as well as for the development of high value-added materials for different applications. The scope of the present review is to perform an overview of important lignins, their isolation and characterization, as well as their representative pharmaceutical applications.

1.   Biosynthesis and Structural Features of Lignins

The biosynthesis of lignin occurs in form of a *post mortem* process in plant cells.(29,30) p-Coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol as the three monolignol building blocks for lignin (**Figure 1**), they are produced presumably from l-tyrosine(31–34) or phenylalanine(35,36) in living cells in proximity to dead cells in the plant, and transported into the dead cells where they are consumed during lignification. While being induced by enzymatic activation of the monomers, the polymerization proceeds most probably without any influence of dirigent proteins. It is still subject to discussion whether other external factors as part of a microenvironment in which the radical coupling takes place can 'guide' lignin formation.(37,38)

Relative abundances of the different lignin monomers vary as function of the type of plant. For instance, lignin of gymnosperms consists de facto entirely of G-type lignin (G-lignin); dicotyledonous angiosperms produce a mixture of G- and S-type lignins (GS-lignin), while monocotyledonous lignin displays all three types of monomers (GSH-lignin).(7,31,32,34)Linkages in lignin are comprised of ether and carbon-carbon bonds between phenolic para-coumaryl alcohol (H-type), coniferyl alcohol (G-type), and sinapyl alcohol (S-type) derived phenyl-propanoid, *i.e.*, the lignin-specific C9 units.(7,31,32)
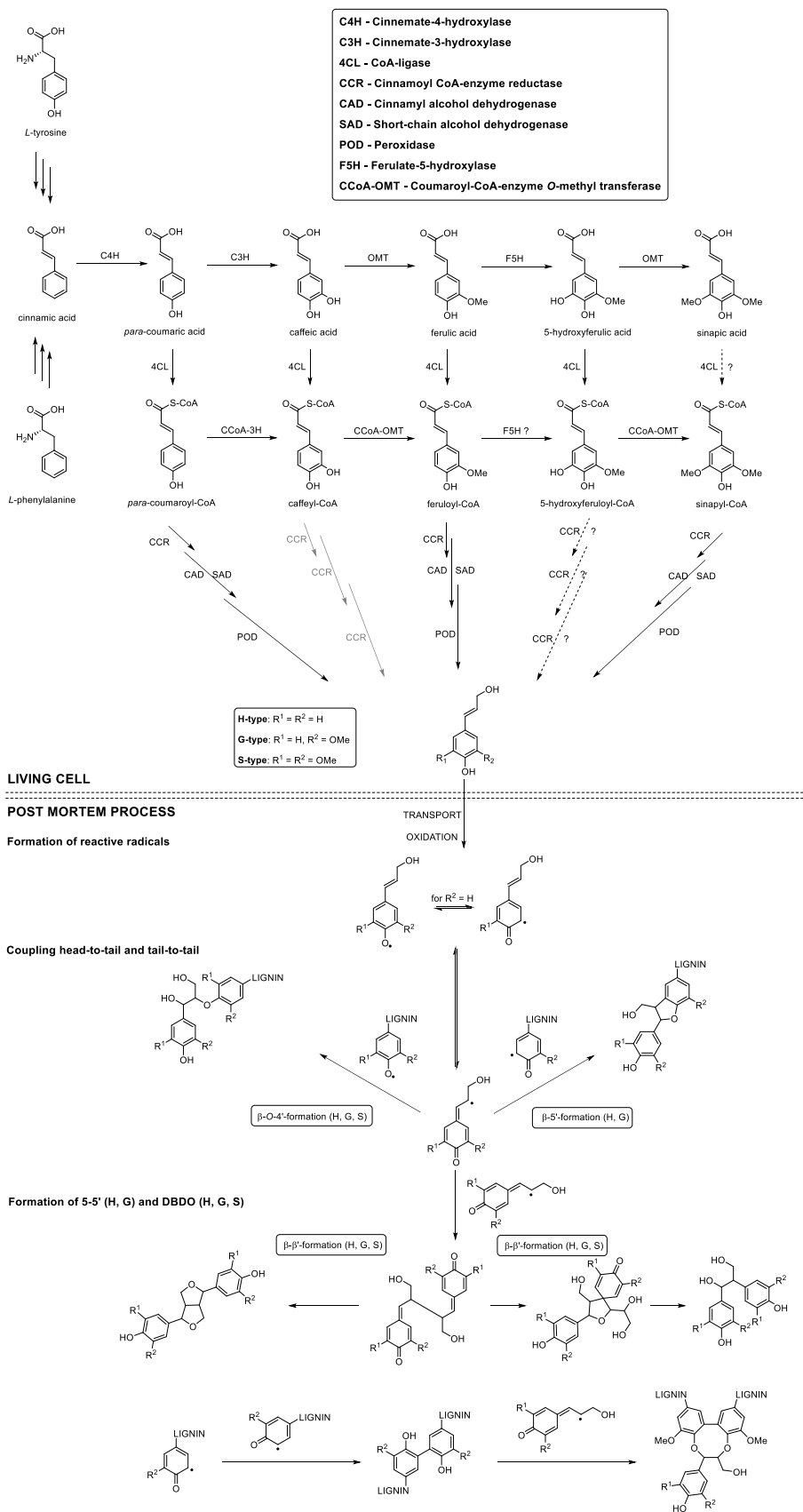
Figure 1: Main elements of the biosynthesis of lignin and formation of typical bonding motifs found in lignins.(38–42)

2.   Important available lignins and lignin fractionation

Different techniques and processes have been established for obtaining isolated lignins, which can be referred to as technical lignins to indicate the differences to native lignin present in the plants (*vide infra*). The most important technical lignin types are: i) kraft lignin (**KL**) obtained from traditional and modified kraft processes,(43,44) ii) organosolv lignin (**OSL**) obtained *via* the Alcell process and variations thereof,(45) iii) lignosulfonate (**LS**) isolated *via* the Howard process.(46)

Any of the above listed procedures, as well as less famous and older traditional processes not listed here used to isolate the aforementioned lignins, often significantly affect the natural structure of lignin, *i.e.*, some of the isolation methods introduce new functional groups and/or cause partial lignin degradation. Isolated technical lignins are thus not necessarily representative of natural lignin polymers. It has now been widely agreed upon in the lignin community, that milled wood lignin (**MWL**), obtained at laboratory scales *via* Björkman's procedure,(47) represents best the natural lignins in terms of the structural motifs present; organosolv lignins are still considered close in their structure to the natural lignin according to current knowledge, while kraft lignin is more drastically changed.(2) Figure 2 shows some representative lignin structures.
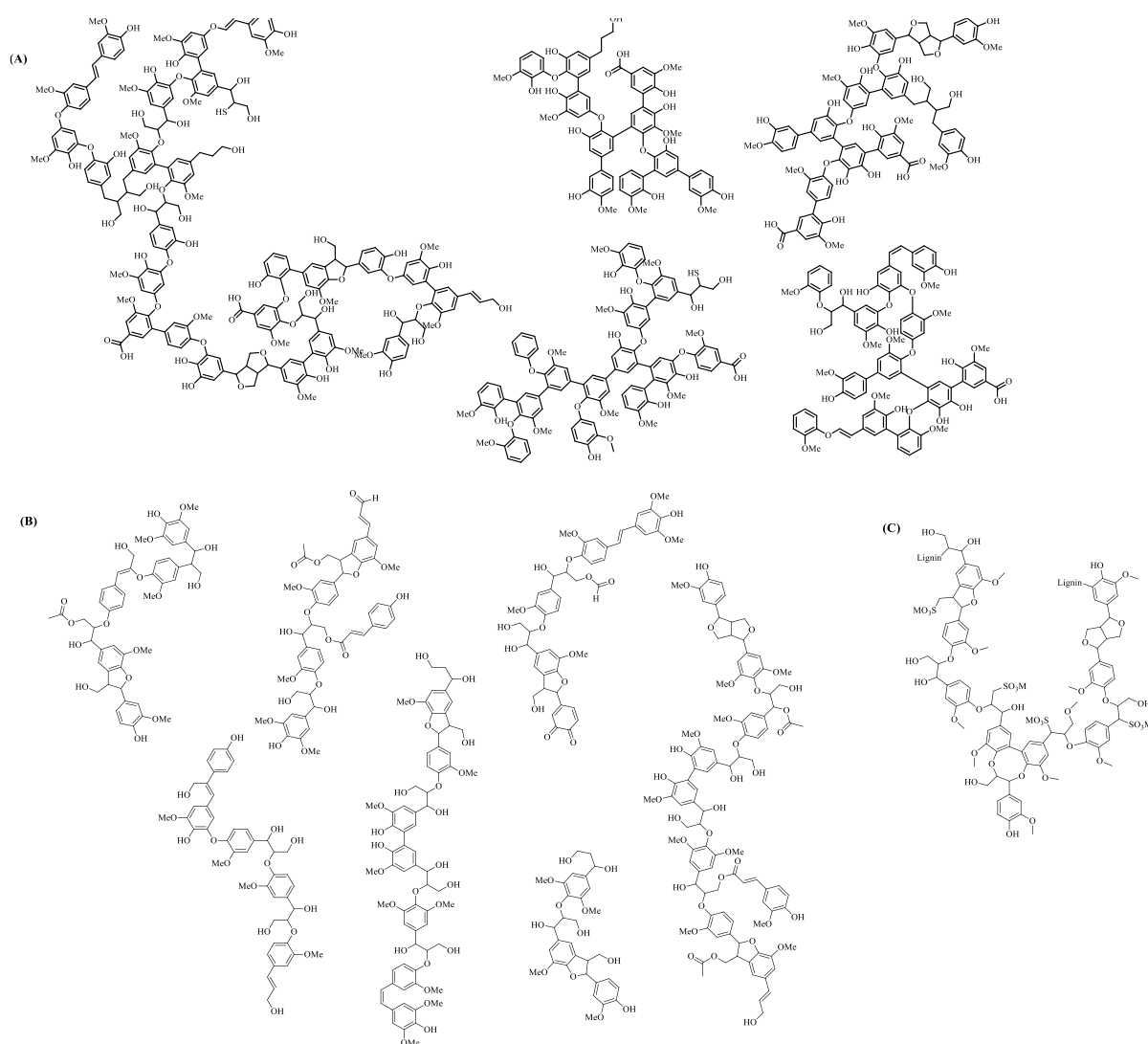

Figure 2: Structural features of lignins isolated: (**A**) kraft lignin (**KL**) isolated from softwood;(48) (**B**) organosolv lignin (**OSL**) isolated from wheat straw;(49) (**C**) lignosulfonate (**LS**) isolated from softwood (common current structural interpretation).

The main challenge in the field of lignin valorisation consists in the structural diversity and heterogeneity across the available technical lignins. Yet the functional groups undoubtedly play a significant role concerning any kind of application, the polymer characteristics as such are important, especially the number average molecular weight (M_n) and the polydispersity index (**PDI**). Indeed, most isolated lignins are characterized by a **PDI** that prevents use in higher value applications, independent of the M_n, which additionally differs significantly depending on the isolation process.

Several efforts aim at the development of lignin fractionation (50–52) and purification processes to obtain lignins and lignin fractions possessing more defined molecular weight ranges, eventually enhanced abundancy in certain functional groups, and divers solubility characteristics.

Common fractionation strategies comprise: i) sequential precipitation out of alkaline solution;(53) ii) fractional precipitation;(54,55) iii) (sequential) extractions using different solvents;(50,53,56–60) and iv) fractionation by ultrafiltration of black liquor using ceramic membranes.(61–63) Some very recent reviews give more extensive overviews and discuss also the challenges that would still eventually need to be overcome.(51,64–70)

Importantly and interestingly, form a regulatory point of view, fractionation of isolated lignins offers the possibility to arrive at repeatable structural features that can be seen as a prerequisite for use in biomedical and pharmaceutical fields.

3.   Physicochemical characterization of isolated lignins

Several analytical methods have been validated for the physico-chemical characterisation of isolated lignins, nowadays. Thus, classical wet-chemical and spectroscopic techniques have been used to provide qualitative and quantitative information on functional groups and linkages of constituents in lignin as well as the degradation products.

Classical wet-chemical analyses are often destructive for the lignin backbone. This drawback is overcome through modern techniques, especially modern nuclear magnetic resonance (NMR)-based techniques that allow an analysis of isolated lignins without touching the oligo- or polymeric structure.

The structural understanding is mandatory with respect to insights into observed/intended physiological actions of lignin and their eventual use in pharmaceutical applications. Some these aspects more recently achieved are highlighted in this work, while more information is available in more dedicated literature resources cited.(65,71–76)

3.1.   NMR spectroscopy-based analysis methods

More insight into structural information can be obtained *via* NMR spectroscopy-based analysis methods, since NMR spectroscopy allows obtaining both detailed structural information and eventual quantization of data. Accounting for the importance of NMR analysis for the structural understanding of lignin, several very good reviews and monographs have been written by pioneering experts in the field.(66,67) The main difficulty hampering the analyses using NMR methods is an eventually low solubility of the technical lignins in the typical NMR solvents, *e.g.*, deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (DMSO-d_6). Solubility issues can be circumvented by acetylating the technical lignin before analyses,(68,76) accepting the fact that the observed shifts are affected by the presence of the acetyl groups. Especially useful for structural analysis of isolated lignins are ^{1}H-^{13}C HSQC measurements of sufficiently concentrated samples, since it allows to identify the interunit bonding motifs in **Figure 1**, and to delineate at the same moment eventually present impurities in form of extractives, sugar residues, etc.(72) Quick-quantitative heteronuclear single quantum coherence (QQ-HSQC) is a method for the acquisition of quantitative HSQC spectra, but often the available NMR equipment dictates the method that should be employed. In case a high-field (400 MHz or more) NMR machine equipped with a cryo-probe is available, either a QQ-HSQC pulse sequence,(69) or an approach consisting of a series of HSQC-measurements with incremented repetition times and mathematical backward-extrapolation, called HSQC$_0$,(70) can be adopted for the acquisition of quantitative two-dimensional lignin spectra.(11,49,77) Another option is the acquisition of high quality standard ^{1}H-^{13}C HSQC spectrum and achieve its quantification of the structural motifs based on a quantitative ^{13}C NMR analysis of the same sample that was used for acquisition of the HSQC spectrum, given the sample exhibits the necessary structural stability in the solvent of choice in the time frames needed for the separate measurements.(78)

Both the number and the nature of free hydroxyl groups can be determined using ^{31}P NMR spectroscopy on phosphitylated lignins in the presence of an internal standard; quantitative ^{31}P NMR is one of the few standard analytical tools in lignin chemistry.(76) This method represents perhaps the most reliable, facile, and diffused analytical technique for the fast quantitative and comprehensive characterization of the most important structural and chemical features of lignins. Advantages in the use of ^{31}P NMR lie in the low amount of sample needed, reduced analysis time, ease in sample preparation, and reproducibility. The usefulness of quantitative ^{31}P NMR can also be seen from the contribution it plays to the overall characterisation of a lignin sample.

3.2. Size exclusion and gel permeation chromatographic methods

A more promising way for determining molecular weight key features of lignins, such as mean average molecular weight (M_n), weight average molecular weight (M_w) and the PDI is the use of size exclusion chromatography (SEC) or gel permeation chromatography (GPC).(79,80) These methods, however, suffer from problems emerging from the diversity within the chemical structure of lignins. Namely, the structural differences between different lignins theoretically require a set of tailor-made standards for achieving a calibration of the SEC setup that fits the characteristics of the lignin analyte. It has been shown that universal calibrations do not lead to better results than calibration only based on commercially available polystyrene standards, thus indicating that differences in the hydrodynamic volume cannot be the only issue to be considered.

Most interestingly, newer data suggest that at least isolated lignins are much less polymerized as anticipated in the past. Hence, isolated lignins exhibit polymer sizes that fit the dynamic range of more distributed methods such as SEC and GPC,(81,82) which have been even adopted for constant quality monitoring purposes.(79)

3.3. Fourier-transform infrared spectroscopy

The moderate financial requirements for the experimental equipment and the wide-spread use of Fourier-transform infrared spectroscopy (FT-IR) also in other fields related to lignin research contribute to IR-analysis maintaining its importance in the field. Different sampling techniques beyond the traditional and most often-used potassium bromide (KBr) pellet are available and applicable in the lignin field, and measurements are also done by default using the nowadays more common attenuated total reflectance (ATR)-IR.(83)

3.4. UV-vis spectroscopy

While UV-vis spectroscopy is used by default in the determination of total lignin content and the determination of antioxidant activities, it also offers the possibility to determine certain structural features, given that the lignin under analysis is sufficiently soluble. Based on the theory that the spectra of phenolic groups present in lignin backbones can be seen as overlays of a shifted version of the three standard bands of unsubstituted benzene,(84,85) the three different types of phenols, *i.e.*, H-, G-, and S-types present in a sample can be delineated in combination with extensive model studies. A historical survey of the origin of today's high standard(87) has been accomplished before.(86) As highlighted by several studies, knowledge of the respective extinction coefficients is necessary to obtain detailed and quantifiable data,(87) but practical results continue to suffer from the fact that isolated oligomeric or polymeric lignins that are additionally possibly polluted with UV-active species exhibit a complexity in the UV-spectra that is more than the sum of its monomers. This problem is also apparent in the various works that employ difference spectroscopy for the quantification of phenolic groups, ethylenic double bonds, non-condensed phenolic groups, and phenylcoumarans.(88–90) Specifically, a correlation between the number of total phenolics determined *via* UV difference spectroscopy with the total phenolic content determined *via* quantitative ^{31}P NMR spectroscopy is not necessarily always given.(86,91) Nevertheless, UV-based determination of phenolic groups according to standard protocols remains a valid alternative for comparative studies.

3.5. Antioxidant activity

Antioxidant activity is one of the most important properties of lignins,(92–99) especially for envisaged applications,(97,100) and is traditionally measured in dedicated assays based on UV-spectroscopy. Established methods that are ultimately based on UV-vis spectroscopy include Total Radical-Trapping Antioxidant Parameter (**TRAP**),(101) Oxygen-Radical Absorbance Capacity (**ORAC**),(102) superoxide radicals scavenging,(103) the 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) method,(104) and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (**ABTS**) radical cation assay.(105,106) Most of the methods express activity of the sample as standard antioxidant equivalents per gram of freshly weighted samples. Gallic acid (**GAE**), tannic acid (**TAE**), ascorbic acid (**AAE**) and others can be used as standard antioxidants. Solubility issues are eventually encountered especially when analysing lignins, so that occasionally emulsifiers might be used to ensure full sample dissolution.

4. Promising properties of Lignins for Pharmaceutical Applications

Lignin shows very interesting properties from a material's point of view in a broad range of areas.(6,107,108) With respect to exploitation in the pharmaceutical and/or biomedical field, especially as a leading ingredient, the situation is very different with respect to other fields of application.(109–112) Despite years of research in the field, this situation is still changing only very slowly, a fact that is indicative a series of challenges that are intrinsic to the biopolymer lignin, and that are connected not only to scientific aspects, but also emerge with respect to current regulatory guidelines. Nevertheless, since the structural analysis of

lignins is maturing, and fractionation protocols applied to isolated lignins are about to allow for a narrowing down of structural diversities in generated fractions, problems can be expected to diminish significantly. Additionally, both chemical and biotechnological routes to tailored lignins are subject to current research efforts that are basically suitable for rendering lignins more, or more easily suitable for pharmaceutical applications (109).

4.1.   *Lignin as source of, or as pharmaceutical active*

Lignins can be considered interesting substances with respect to anti-tumour treatments;(113) in numerous studies were investigated: i) polyphenols with structural motifs found in lignin; ii) mixtures of the monomers lignin is composed of; or iii) small aromatic molecules that could be the enzymatic degradation products of lignin, in order to evaluate the response of tumour development and growth and the effects on underlying and signalling pathways to these potentially lignin-derivable substances.(114,115) More classical structure-activity relationship studies in the classical sense are difficult, some initial trials exist and have been recently discussed;(113) the challenges in this context can be understood in light of the difficulties that would be encountered in correctly describing the regularly irregular poly- and oligomeric lignin structure *in silico*. Often, the observed cytotoxic activity of lignin and lignin derivatives is the result of interactions of lignin with other substances such as ascorbic acid,(116) or lignin containing natural complexes such as lignin–carbohydrate complexes (**LCCs**).(117)

controversially discussed in detail still in terms of structure and general natural occurrence **LCCs,**(118–124), have been tested in various more or less isolated forms with respect to a series of pharmacological activities, including antiviral, antibacterial and anti-inflammatory effects.(118–124) The effects are hence the same as those found for some lignins. Since **LCC** motifs are commonly found in isolated lignins as function of feedstock and biorefinery method, it is difficult to judge whether the observed activities stem from the polyphenol part alone or from the connection between lignin and carbohydrates. Since the described activity profiles are normally not attributable to carbohydrates, it seems most likely that a polyphenolic moiety plays a major role. More research is necessary, however, using a very pure isolated sample of **LCCs**, obtainable, *e.g.*, *via* fractionation of isolated lignins,(125–127) in comparison to **LCC**-free polyphenol preparations to arrive at general structure-activity relationships.

Comparably few studies exist that see lignin, or derivatives thereof, as the main pharmaceutically active ingredient as discussed above; its role consists yet much more often in furnishing simple beneficial actions, such as antioxidant,(93–99,128,129) anti-inflammatory,(17) or UV-shielding activities that exert their potential in form of synergistic interactions with the active, or as protection for the active itself.(18,64,130–132)

Reports exist on research activities focusing on potential anti-viral activities of lignin derivatives, also against HIV reported.(19,20)

4.2.   *Lignin as matrix-element in preparations*

5.2.1 Lignin as 'classical' excipient

On the basis of these possibilities, and despite the structural challenges with respect to regulatory aspects, the suitability of lignin as excipient for the production of conventional tablets has been tested reported.(20,133–135) Incorporating standard actives such as acetylsalicylic acid or paracetamol, the presence of lignin or chemically derived carboxylated lignin led to altered release profiles for the active ingredient; generally, the presence of lignin increased the release efficiency compared to controls, while protecting the active.(134)

5.2.2 Lignin as an ingredient in the formation of hydrogels

Hydrogels have existed for more than half a century, and today they have many applications in various processes ranging from industrial to biological. The hydrogel can be defined as three-dimensional (3D) network of hydrophilic polymers that can swell in water and hold a large amount of water while maintaining the structure due to chemical or physical cross-linking of individual polymer chains. Hydrogels have been one of the promising candidates in tissue engineering due to their extended polymer network being capable of mimicking the surrounding tissue environment.(136)

The structural features of lignin, and the resulting physico-chemical properties, render it a promising natural biocompatible polymer in the development of hydrogels for tissue engineering. In this respect, alkali lignin as an ionotropic cross-linker, together with chitosan, has been used to generate chitosan-alkali lignin hydrogels, which were proven to be non-cytotoxic when tested *in vitro* against Mesenchymal stem cells and zebrafish.(137) Similarly, lignin extracted from coconut has been integrated into a thermo-responsive polyurethane-based nanogel for wound-dressing application.(133) For instance, *in vivo* studies using mouse burn wound models demonstrated the wound healing potential of the nanogel.(112) In addition, the hydrogel exhibits antioxidant capability. Further *in vivo* investigations were conducted on burnt and infected wounds of rats. Results revealed

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promising abilities such as infection prevention and wound healing capabilities.(112,134,137) In summary, lignin was proven to be a viable ingredient in the formation of lignin-based hydrogels. Such hydrogels in the various studies were capable of wound healing with no cytotoxic effects. Hence, making them a potential biocompatible material in the field of regenerative medicine.(112)

Table 1: Summary and key findings of lignin-containing hydrogels.

Technical lignin	Matrix material / additives[a]	*In vitro* study	*In vivo* study	Key findings	Year (Ref.)
Kraft lignin	Hyaluronic acid	mouse embryonic fibroblasts	-	Non-cytotoxic (>90% cell viability); positive cell migration and cell growth over 2–7 days	2018 (134)
Kraft lignin	**PLA** / Rose Bengal, gold silver	*Candida tropicalis*	-	Antifungal and antimicrobial properties at very low IC_{50} values (0.1 μg/mL)	2020 (135)
Lignosulfonate	**PVA** / chitosan	*Staphylococcus aureus*, bovine serum albumin, mouse osteoblasts	female kunming mice	70% reduce in free radicals with 0.2 mg / mL lignin solution; increased cell viability with increasing lignin solution concentration (0.2 -3.2 mg / mL); good antibacterial abilities at 10 %(w/w) lignin; faster and complete wound healing over 15 days	2019 (138)
Lignosulfonate	Polyoxazoline / triazoles (linked covalently)	Mouse RAW 264.7 macrophage cells *Escherichia* coli - *Pseudomonas* aeruginosa - *Salmonella* typhi *Klebsiella* pneumonia - *Staphylococcus* aureus - *Staphylococcus* epidermidis *Candida* albicans *Candida* tropicalis	Sprague dawley rats	Antioxidant, cellular anti-inflammatory, and antimicrobial activities; capacity of preventing infection; promotion of healing;reduced inflammation on burn wound within 14 days	2017 (139)
Lignosulfonate	**PVA** / silver	*Staphylococcus* aureus *Escherichia* coli Mouse L929 fibroblasts	-	Improved antibacterial properties; non-cytotoxic; biocompatible (≈60% cell viability)	2019 (140)
Alkali lignin	Chitosan	Mesenchymal stem cells Mouse NIH3T3 fibroblast cells	Zebrafish embryo	Biocompatible *in vitro* (99 ± 3% cell viability); positive wound healing potential after 24 h; low cytotoxicity up to 100 μg/mL; suitable cell attachment; proliferation, and migration surface	2019 (137)

Alkali lignin	Carrageenan / calcium chloride, copper chloride magnesium chloride silver	*Staphylococcus* aureus *Escherichia coli* Mouse L929 fibroblasts	Sprague Dawley rats	Antibacterial behavior between 3 and 6 h Biocompatible properties (>95% cell viability after 48 h) Wound healing effect within 14 days	2020 (141)
Organosolv lignin	**PEG**, **PPG**, **PDS**	Human L-02 hepatocytes	SPF mice	Excellent biocompatibility (>90% cell viability); cell survival rate of 64–83% in the oxidative stress cell model; rapid and complete wound healing within 25 days	2020 (133)
Lignin-carbohydrate complex	**PEG** diglycidyl ether	Human L-02 hepatocytes	-	Positive hepatocytes adhesion; biocompatible with cell proliferation and metabolic activity (highest after 5 days)	2017 (142)

a: **PLA** – poly(lactic acid); **PVA** – poly(vinyl alcohol); **PGS** – poly(glycerol sebacate); **PCL**-poly(caprolactone); **PEO** – poly(ethylene oxide) ; **PEG** – poly(ethylene glycol); **PPG** – poly)propylene glycol); **PDS** – poly(dimethylsiloxane).

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4.3. *Lignin as material for gene and drug delivery in micro- and nanocarriers*

Drug and gene deliveries are used in both targeted delivery and controlled realise of therapeutic agents which can improve the efficiency of the therapeutic agents.(112) In this respect, lignins, to some extent, can be used as a valid alternative of tannins, in the generation of hydrogels and microcarriers, *i.e.*, micro- and nanoparticles, micelles or capsules. In fact, their intrinsic properties offer additional benefits when these natural products are used in the generation of carrier systems for biomedical applications.

Reports exists in which lignins have been used to produce capsules capable of encapsulating either hydrophobic(21,23) or hydrophilic drugs.(24) This fundamental principle could be extended to various lignin types, realising a pH-dependent release mechanism for entrapped actives.(23)

Given the interesting and well investigated properties of lignin in the fields of material science, in combinations with especially the aforementioned UV-shielding and antioxidant properties, the use of lignins for the fabrication of particles, capsules and films in nano- and microscales is attractive. Being currently a very active field of research, several recent reviews exist that highlight the various recent progresses.(111,143–148)

4.3.1. *Lignin-based nanotubes in gene delivery*

Synthesis of lignin nanotubes has been conducted using aluminium with interpenetrating holes as sacrificial templates.(149) The length (11–19 μm) and diameter (about 200 nm) of the nanotubes were influenced only slightly by the different types of lignin raw materials used. For the synthesis of lignin nanotubes, the alumina membrane was activated with APTES then Schiff's base was reacted between the amino groups on the APTES-activated membrane and aldehyde moieties in the thioglycolate lignin to form a base layer. Subsequently, hydroxycinnamic acids, hydroxycinnamaldehydes or hydroxycinnamyl alcohols were added and polymerized onto the lignin base layer through a reaction catalyzed by horseradish peroxidase (HRP)/H_2O_2.(149)

To overcome the dose-dependent cytotoxicity of polyethylenimine commonly used in gene transfection, recent advances in gene delivery suggest lignin as potential carrier material. However, lignin on its own lacks a binding site for the negatively charged DNA. In this respect, the use of lignin-based nanotubes and functionalization of lignin with potential DNA binding sites were investigated and the potential of using lignin nanotubes (**LNTs**) as a gene delivery vehicle has been demonstrated.(22) The formulated **LNTs** were able to enter HeLa cells without the need for auxiliary agents and they were shown to be able to enter the cell nucleus. They were also capable of binding to DNA and delivering DNA into the nucleus, making them particularly useful in smart gene delivery systems. However, the immunogenicity of using **LNTs** needs to be further investigated, along with the effect of different lignin sources on the immune response.(22)

In addition, lignin-based copolymers have been investigated and they show a high transfection efficiency which is comparable or much higher in respect to the commonly used polyethylenimine control when tested in HEK 293T and Hep G2 cell lines. The copolymer also showed excellent antioxidant activity which made the cationic lignin-based copolymer a suitable candidate for gene delivery.(150)

Caicedo *et al.* functionalized **LNTs** with avidin for specific immobilization onto desthiobiotin-grafted glass surfaces, for application as carriers of bioactive compounds to specific target cells or organs within the human body.(149)

4.3.2. *Micro- and nanosized particles*

Three main routes have emerged for generating lignin nanoparticles (**NPs**). The most common methods for the preparation of lignin **NPs** involves solvent exchange by adding an anti-solvent to a solution of lignin, or *vice versa*, resulting in the formation of solid spheres (**Figure** 3 A-C).(151) Aqueous and non-aqueous tetrahydrofuran,(151,152) dioxane,(153) dimethyl sulfoxide,(154,155) acetone,(154,156,157) and ethanol(158,159) have been used for solubilized the starting lignins. Pure water served as anti-solvent in most cases.

Sodium *para*-toluene sulfonate (**Na-PTSA**) was used in an autotrophic approach to generate lignin nanoparticles (**Figure** 3 D).(160) These precipitated upon rapid dilution of an acidic aqueous solution containing kraft lignin and **Na-PTSA** with water.

Non-spherical particles were obtained also in a ultrasonication-based formation of lignin nanoparticles (**Figure 3** E), starting from an aqueous solution of lignin.(161)

Lignin nanoparticles show the antioxidant and UV-protective properties that are typical for the starting lignins.(162) This finding goes together with elucidations that confirm that the lignins remain chemically, *i.e.*, structurally unaltered.(160,163) The latter aspect is especially important with respect to regulatory issues and an eventually guaranteed biodegradability of the lignin part of any pharmaceutical product on the basis of lignins.

4.3.3. *Micro- and nanosized capsules*

With respect to applications in the pharmaceutical sector, usage of lignin particles as delivery vehicle is most obvious; such actives would have to be co-precipitated upon particle formation, requiring a comparable solubility profile. Restrictions that emerge from this aspect can eventually be circumvented using core-shell structures, *i.e.*, capsules rather than solid particles.

Oil-in-water emulsions have been used as templates for the preparation of lignin microcapsules (0.3–1.4 μm) by ultrasonication (**Figure 3** F).(21,163–169) Applying the same approach, generation of lignin-metal frameworks as shell material was demonstrated as well.(23) Ultrastirring of lignin-containing emulsions has been demonstrated to be applicable to lignin micro- and nanocapsules formation (**Figure 3** G).(76)
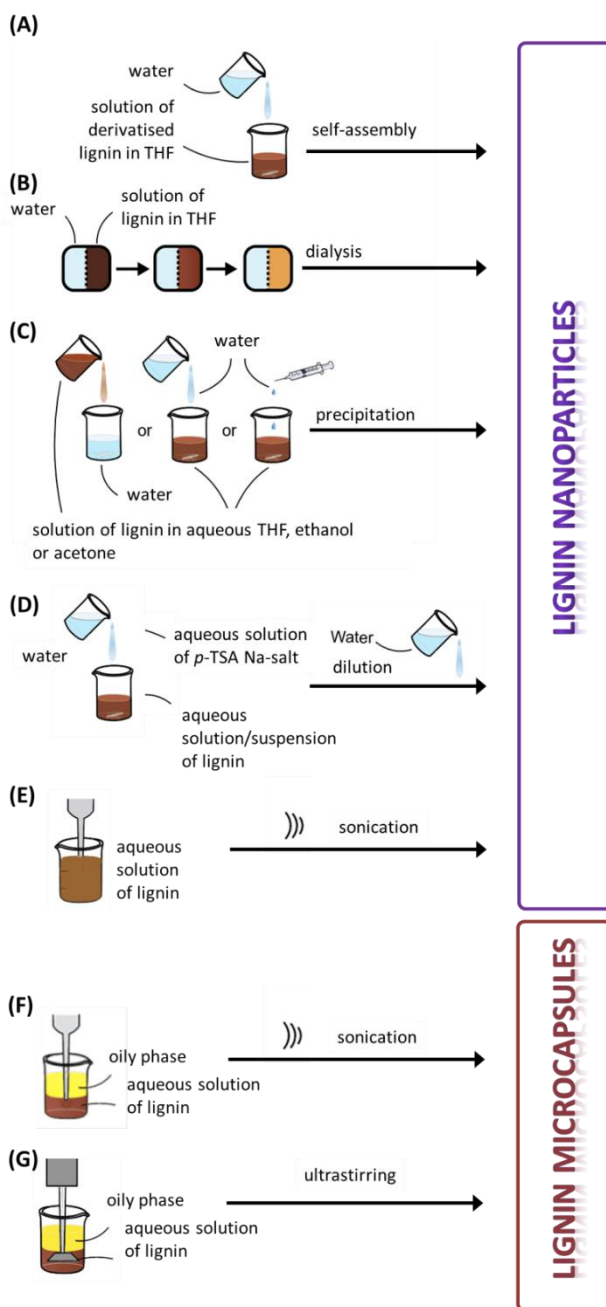
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Figure 3: Straight forward fabrication modes for generating of nano- and microscale lignin particles and capsules: (A) adding water into THF solution of suitable or suitably modified lignin; (B) dialysing a solution of suitable lignin in THF against deionised water; (C) nanoprecipitation by adding water into a solution of a suitable lignin; (D) formation of lignin nanoparticles using a hydrotropic system; (E) formation of non-spherical lignin nanoparticles upon ultrasonication of aqueous lignin solutions. (F) ultrasonication of emulsions of an oil and an aqueous solution of a suitable lignin; (G) ultrastirring of an emulsion of an oil and an aqueous solution of a suitable lignin.Refer to main text and Table 3 for references. Figure realised in part on the basis of reference(143).

4.3.4.   Incorporation of actives in lignin particle and lignin capsule structures

The typical characteristics of lignins in terms of UV-shielding and anti-oxidant properties can be exploited in the use of the above-described lignin particles and capsules as green delivery vehicles in various fields of application. Such carrier systems are capable of trigger-related slow or fast release of entrapped or encapsulated actives, which are protected from external, eventually degrading factors such as UV-radiation or molecules. *Vice versa*, biological and or environmental matrices in which particles and capsules will be eventually immersed are protected from the active ingredients unless matrix characteristics change to trigger slow or fast particle and capsule decomposition.

Entrapment, encapsulation, adsorption, and covalent binding are common methods for loading actives into lignin materials (**Figure 4**). The loading of the cargo may take place during or after the formation of nanoparticles and nanocapsules, by entrapment or encapsulation, respectively. The main hurdle is in both cases a sufficient, compatible solubility of the active, which in the case of generations from biphasic systems can become challenging given the ultimately necessary hydrophilicity of most actives.
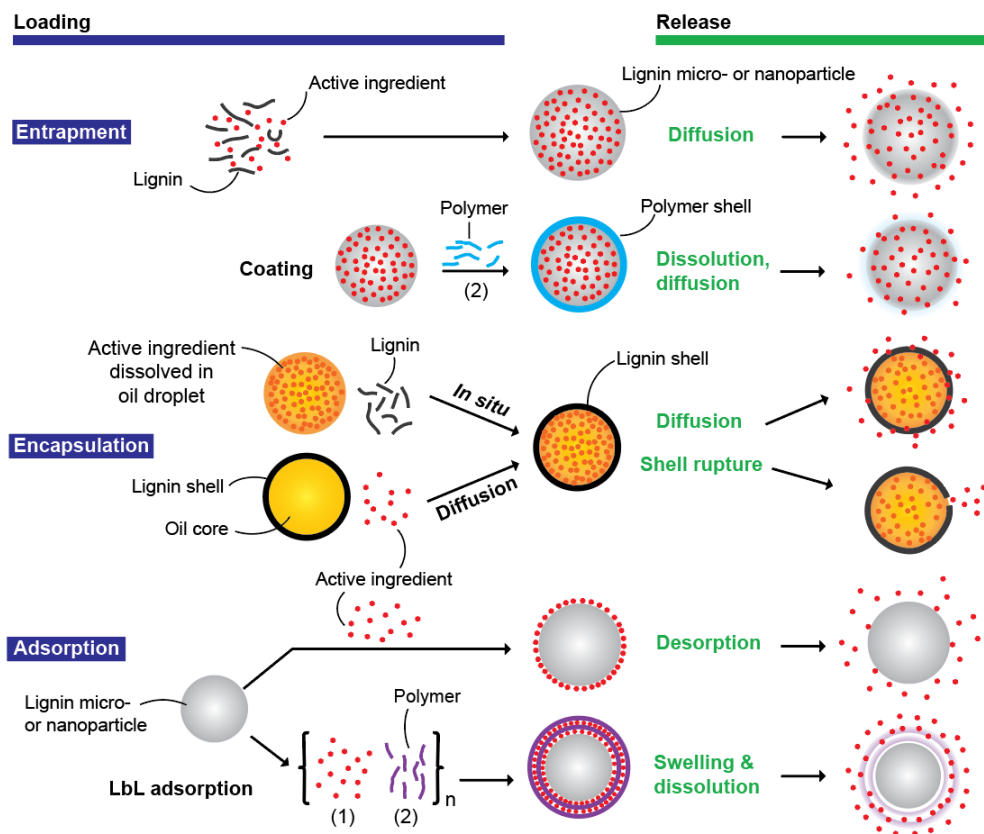

Figure 4 : General methods for loading and releasing actives from a variety of different carriers. Used with permission from reference (143), copyright (2019) Wiley VCH.

4.3.5.   Entrapment vs. encapsulation vs. adsorption

Entrapment is the most common method for incorporating small active molecules into solid lignin particles. The current view is that lignin **NPs** form by the supramolecular assembly of poorly water-soluble molecules *via* electric interactions with aromatic rings.(151,163,170) As could be expected from this mechanism, most of the substances currently successfully entrapped in lignin **NPs** are poorly water-soluble lower molecular weight compounds. A couple or pioneering

reports describe the loading of pharmaceutically relevant small molecules and biological macromolecules such as antibodies and enzymes, as reported in Figure 5.

Encapsulation can be used to dissolve lipophilic drugs in the water-immiscible cores of capsules dispersed in aqueous media, and *vice versa*. The ratio of sphere volume to surface area equals diameter/6, and therefore microcapsules appear more efficient than nanocapsules if compared solely based on the volume available for loading of the active. However, nanocapsules with diameters of around 200 nm have exhibited benefits over microcapsules in drug delivery to cancer cells.(2,59,76)

Immobilization of macromolecules, including oligopeptides and enzymes is normally achieved *via* covalent surface-linking or simple electrostatic adsorption. Yet this method of incorporation of actives in the context of lignin-based particulate and capsule systems represents a smaller fraction in the context of lignin-based carrier systems, and has been used only for enzymes in pharmaceutically relevant applications: in order to increase enzyme stability, lipase was adsorbed on composite material consisting of chitin and oxidized kraft lignin,(76) while cutinases and porcine pancreatic α-amylase were adsorbed on acetic acid lignin.(171)

Figure 5 reports a selection of recent works in which lignin-based particulate or capsule systems have been employed for supporting actives relevant to the pharmaceutical sector. The relatively small number of entries compared to the overall rather high number of publications in the lignin particle and lignin capsule sector, as well as patents detailing synthesis and potential use of lignin nanoparticles and microcapsules in general, can be seen as indicative of the challenges technically connected to the biomedical fields of application.
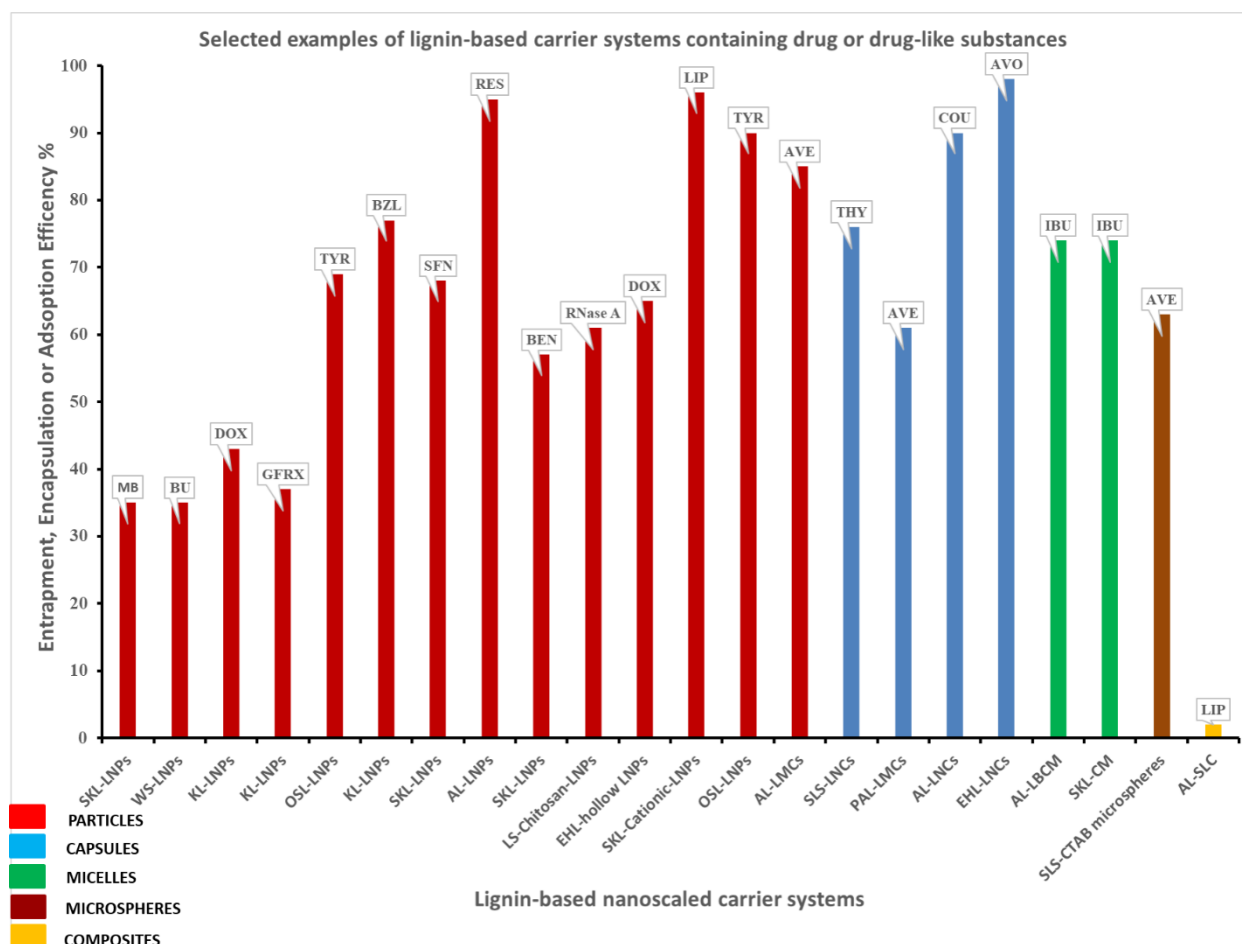

Figure 5: Micro- and nanoscaled particle, capsule, micelle and composite systems based on lignin for use as carrier systems. Selected examples report incorporation of drugs or drug-like substances. **MB**, methylene blue; **BU**, budesonide;

DOX, doxorubicin; **GFLX**, gatifloxacin; **TYR**, tyrosinase; **BZL**, benzazulene; **SFN**, sorafenib; **RES**, resveratrol; **LIP**, lipase; **AVE**, avermectin; **THY**, thymol derivatives; **COU**, coumarin 6; **AVO**, avobenzone; **IBU**, ibuprofen.

4.3.6. *Covalent and electrostatic surface functionalisation*

Chemical modifications of base material used for carriers and grafting of biologically relevant functional motifs are common methods to render micro- and nanoscaled structures more suitable for an intended application. Compared to the abundant literature on the preparation of lignin-based nano- and microstructures as such, reports regarding their surface modification is relatively scarce. Figueiredo *et al.* succinylated softwood kraft lignin and used the product to generate carboxylated lignin particles (**CLNPs**) that were further modified by chemically attaching a block copolymer comprising poly(ethylene glycol) (**PEG**), poly(histidine) and a cell-penetrating peptide.(172)

Anionic **LNPs** have been modified by adopting a layer-by-layer (LbL) technique generating a layer of cationic polymers such as poly(diallyldimethylammonium chloride) on their surfaces.(170,173) This simple possibility allowed for amplifying the potential application range of the particles. Using a variation of the LbL-approach, adsorption of chitosan on **LNPs** was shown to form colloidally stable cationic particles that fully stabilised olive oil-in-water emulsions.(174)

Antibacterial silver nanoparticle composites enclosed in a matrix of poly(vinyl alcohol) and lignin isolated from spent pulping liquor were reported, to arrive at nanomaterials that exhibit a boosted antibacterial activity.(175) Silica/lignin hybrid particles comprising silver **NPs** have been reported for the same purpose.(176) Commercial silica material was modified with *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane to increase affinity to kraft lignin oxidised with sodium periodate. Silver **NPs** grafted onto the resulting silica/lignin hybrids were stable and active against *Pseudomonas aeruginosa*. Silver-ion-containing lignin nanoparticles have been realised as eco-friendly alternative to silver nanoparticles in antimicrobial applications.(177)

4.3.7. *Lignin-containing film preparations for pharmaceutical applications*

Lignin has been used as ingredient in various film preparations, exploiting its anti-oxidant and anti-inflammatory activities. Films have been developed for applications in wound healing and tissue engineering. **Table 2** lists selected works with the respective references. An incorporation of actives has not yet been reported.

Table 2 : Electrospinning preparation of lignin-containing film for pharmaceutical and biomedical applications.

Lignin source	Products[a]	Field of application	Year (Ref.)
Alkali lignin	**PLA**-lignin fibres	Tissue engineering	2016 (177)
Organosolv lignin	**PEO**-chitin nanofibrils-lignin fibres	Wound healing	2016 (178)
Lignin as decoration for multi-walled nanotubes	**PVA**-lignin fibres	Wound healing/tissue engineering	2018 (179)
Kraft lignin	**PVA-PGS**-lignin fibres	Tissue engineering/regeneration	2019 (180)
Kraft lignin	**PCL**-lignin fibres	Tissue engineering/regeneration	2020 (181)

Challenges in the area of lignin-containing films exist in form of compatibility of lignin with the main matrix polymer, lignin solubility, and often affected mechanical stabilities and/or homogeneities of films when lignin concentrations exceed a certain concentration. While thermal stability often found to benefit from lignin presence, controlling mechanical stability becomes more challenging due to the varying plasticizer characteristics of different lignins. Some reported applications do actually not use lignin as integral part of the fibres, but as nanoparticulate additive during the fibre generation. Most of the publications used kraft or alkali lignins; especially kraft lignin in crude form might expose,

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however, higher risks in terms of cytotoxicity compared to lignins stemming from other biorefinery approaches like organosolv processes.

Conclusion

The exponentially increasing number of scientific reports regarding the use of lignin in pharmaceutical applications indicates that the field is maturing, agreeing on the fact that most benefits can be obtained when using lignin as material for carrier systems. The formation of such carrier systems from essentially all types of currently available lignins can be interpreted as that a technological plateau has been reached, and that the methods for the generation of particles and capsules have become mainstream. Various actives are incorporated, and tuning is possible to achieve stability profiles that would suit specific applications.

However, final proof are missing regarding the real-life applicability of the developed systems. In addition, open questions remain regarding the possibility for lignin-based carrier systems to pass regulatory hurdles. It would also be mandatory to understand the fate of lignins in front of the human metabolic system. It has been suggested that lignins, used in an isolated form or used as an excipient, could be treated as fibres in nutraceutical definition from a regulatory point of view, but given the proven activity of lignin in form of, *e.g.*, anti-inflammatory agent, renders also this approach challenging.

Lignin fractionation seems to be a promising approach to arrive at lignins that might facilitate the regulatory aspects while maintaining the lignin benefits. Additional challenges connected to the use of lignin in nanoscaled preparations lie in potentially adverse, toxic effects that originate from the nanoscale, would need to be investigated and resolved. While eventually non-toxic as bulk material, once lignins are concentrated in form of a nanoparticle or microcapsule, local toxicities can occur due to the unnaturally high concentration of their reactive phenolic groups.

In terms of using lignin as the main active, besides challenges similar to those just discussed, more work in terms of structure-activity relationships is needed that might require an innovative *in-silico* description of lignins.

Conflict of interest

None to declare.

Acknowledgement

None to declare.

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