

Review

Lignin-Derived Biomaterials for Drug Release and Tissue Engineering

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Abstract: Renewable resources gain increasing interest as source for environmentally benign biomaterials, such as drug encapsulation/release compounds, and scaffolds for tissue engineering in regenerative medicine. Being the second largest naturally abundant polymer, the interest in lignin valorization for biomedical utilization is rapidly growing. Depending on resource and isolation procedure, lignin shows specific antioxidant and antimicrobial activity. Today, efforts in research and industry are directed toward lignin utilization as renewable macromolecular building block for the preparation of polymeric drug encapsulation and scaffold materials. Within the last five years, remarkable progress has been made in isolation, functionalization and modification of lignin and lignin-derived compounds. However, literature so far mainly focuses lignin-derived fuels, lubricants and resins. The purpose of this review is to summarize the current state of the art and to highlight the most important results in the field of lignin-based materials for potential use in biomedicine (reported in 2014-2018). Special focus is drawn on lignin-derived nanomaterials for drug encapsulation and release as well as lignin hybrid materials used as scaffolds for guided bone regeneration in stem cell-based therapies.

Keywords: biomaterial, bone regeneration, drug release, hydrogel, lignin, multivariate data processing, osteogenesis, scaffolds, stem cells, tissue engineering

1. Introduction

Materials used in biomedicine, such as polymers for drug encapsulation and tissue engineering scaffolds are preferably produced from natural compounds, such as collagen-based composites for bone repair or alginates for controlled drug delivery. So far, numerous biopolymers have been studied in detail regarding their ability to be appropriate for release materials and/or scaffold applications most of them are designed using polysaccharides, lipids and proteins [1-3].

Due to the development of biorefinery concepts for biomass treatment, starting about ten years ago, lignins gained increasing interest in academic and industrial research. In particular, lignocellulose-rich feedstocks (LCF) are described for energetic and material exploitation [4].

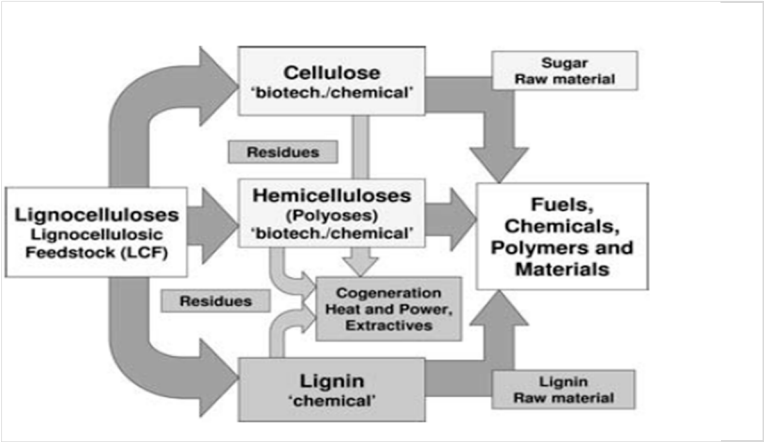


Figure 1. Lignocellulosic feedstock biorefinery. [4] Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

In between, a number of pilot plants have been established and lignins are even commercially available to a limited extent [5-7]. Remarkable progress was made in lignin research, in particular isolation, structure analysis, functionalization and modification. Market analysis studies were published in 2017 and 2018, stating that the global lignin market is predicted to reach an annual growth rate of about 2 % until 2023 and an increasing total market size from US\$ 904.04 Mio in 2017 to US\$ 1021.57 Mio in 2023 [8, 9]. According to the number of published patents and scientific studies, industrial applications so far are mainly directed toward lignin-based additives in concrete, dispersants, binders and resins. In contrast, studies including lignins for biomedical applications (release materials and/or scaffolds) are still very rare (Tables 1 and 2).

Table 1. Number of scientific publications on “lignins” refined by “lignin in drug release” and “lignin scaffolds” according to Web of Science, searched on 2018-06-20.

Publication Years	“lignin”	“lignin & drug release”	“lignin & scaffolds”
2014	2,856	3	23
2015	3,269	5	25
2016	3,672	10	35
2017	3,893	12	39
2018	1,783	8	13

Table 2. Number of patents specifying “lignin”, refined by “lignin in drug release” and “lignin scaffolds”, respectively, according to WIPO, searched on 2018-06-20.

Filing Year	“lignin”	“lignin & drug release”	“lignin & scaffolds”
2014	5877	474	683
2015	5766	440	601
2016	5912	449	601
2017	5264	412	488
2018	1691	153	183

In general, lignin could be used in many fields due to its dispersing, binding, complexing, and emulsion-stabilizing properties. However, lignin valorization is still challenging due to its complex and irregular chemical structure. Thus, the upgrading of lignin-derived materials toward applications in biomedicine is still limited to a very few examples. It requires much effort particularly regarding reproducible quality of the isolated structures. Today, sequential depolymerization via oxidative or reductive methods is one of the favored approaches to generate well-defined lignin fragments. In 2018, Sells *et al.* comprehensively reviewed the status quo of lignin depolymerization and upgrading approaches [10]. Among the few recently published studies on lignins in medicine are those of Vinardell and Santos: Vinardelli *et al.* focused the bioactivity including antiviral and antimicrobial activity of lignins and their derivatives with special focus on their beneficial effects on human health [11]. Santos *et al.* reviewed recent developments to design and fabricate lignin-based nanostructures for biomedical applications [12].

The purpose of this review is to distinguish the lignin structural and morphological characteristics to be exploited in stem cell-based approaches in regenerative medicine. A special focus is drawn on biomaterials (scaffolds and drug release materials) used for mesenchymal stem cell (MSC) differentiation toward cardiovascular or bone tissue. Although a broad variety of scaffolds and release materials were studied *in vitro* and *in vivo* regarding their capacity to support tissue regeneration, there are just a very few studies including lignin-derived biomaterials so far. In contrast, numerous other natural biopolymers are studied in detail as well as synthetic polymers, glasses, ceramics, hydroxyapatite-based composites and nanostructured hybrids fabricated via conventional and additive manufacturing techniques [13-15].

2. Lignin Availability and Structure

2.1. Lignin Availability

The assessment of the biomass availability and quality is an important first step toward utilizing biomass for the development of value-added chemicals. Countrywide assessments of biomass resources have been performed for many single countries, i.e. U.S., Jordan, Malaysia, Turkey, China, India, Bangladesh [16-18]. However, there are no systematic studies regarding world-wide availability of lignocellulose-rich biomass so far. One of the most important challenges is the handling of multiple biomass feedstock streams. A key step in processing lignocellulosic biomass is the separation of sugars from the lignocellulose. Several pre-treatments are applied for this: physical (grinding, milling), chemical (using acidic or basic aqueous media or ionic liquids), physicochemical (steam, hot water or ammonia fiber expansion) and biological fragmentation (via enzymes, fungi). Currently, the development of LCF biorefineries corresponds to the raw material available and focuses on a complete separation of the cellulose, hemicellulose, and lignin fractions using combinations of mechanical, chemical, and biotechnical methods (Figure 2).

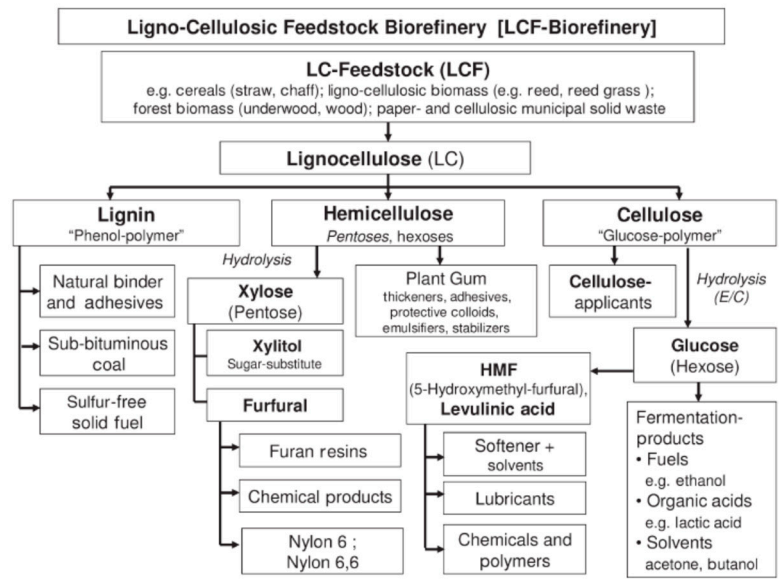


Figure 2. Products of a lignocellulosic feedstock biorefinery. [7] Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Isolation methods such as Organosolv, acid, or alkaline steam-pressure processes are well-known methods which are commercially applied as the Organocell®, Alcell®, or Soda® method [7]. Studies comparing different pulping and isolation techniques could show that the Organosolv process might be the favored one to obtain lignins of good solubility and narrow molecular weight distribution [19-21].

Today, a number of pilot and demonstration plants have been developed using lignocellulosic feedstock (e.g. woodchips, straw) for the production of biobased building blocks (i.e. ethanol, acetic acid) and green coal in Germany (Leuna), The Netherlands (Bioprocess Pilot Facility), Austria (bioCRACK), Canada (GreenField), U.S. (Enchi Corp.) and Australia (Microbiogen) [22, 23]. In addition, first commercial biorefineries are established mainly to produce ethanol from LCF (e.g. Dupont in Nevada, Liberty™ Technology in U.S., POET-DSM in Iowa, Iogen Corp. in Canada, Raízen/Iogen in Brazil, Cellulac in Ireland) [24, 25]. Currently, the pulp and paper industry produces largest quantities of lignin (ca. 55 x 10⁶ tons per year). The most important industrial paper technology is the Kraft pulping process, leading to sulphur-containing degraded lignin fractions which are predominantly used as a secondary energy source. According to the market study, industrial producers of lignin and lignin-derived products around the world include the following: Domtar Corporation (southern pine-based BioChoice®), LignoTech Florida LLC (southern yellow pine-based lignin utilizing a coproduct of RYAM's sulphite pulping process, Borregaard's technology), Weyerhaeuser Company (collaboration with Lignol Energy Corp.), Stora Enso (Lignoboost™, Kraft lignin Lineo™), GreenValue SA (sulfur-free lignin isolated from wheat straw, aqueous alkaline extraction), West Fraser, Domsj Fabriker (world's 2nd largest producer of powder lignin), Changzhou Shanfeng Chemical Industry Co. Ltd. (lignin polyether polyols), Nippon Paper Ind. Co. Ltd., and The Dallas Group of America, Lignosulfonates [9].

2.2. Lignin Structure

Lignin is a complex and irregular biopolymer of randomly crosslinked phenylpropanoid units (cumaryl, coniferyl, sinapyl) identified in plant secondary cell walls. Based on these monolignol units, the lignin building blocks p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) are formed (Figures 3 and 4).

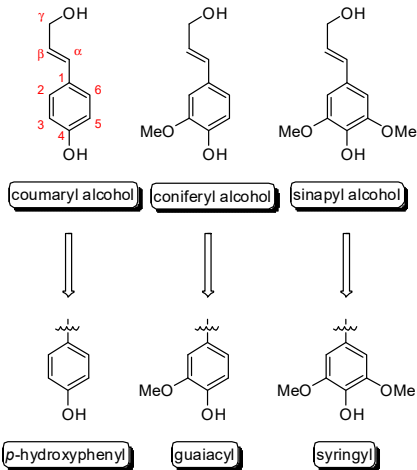


Figure 3. Structure of the three monolignol precursors and their corresponding fragments in the macromolecules.

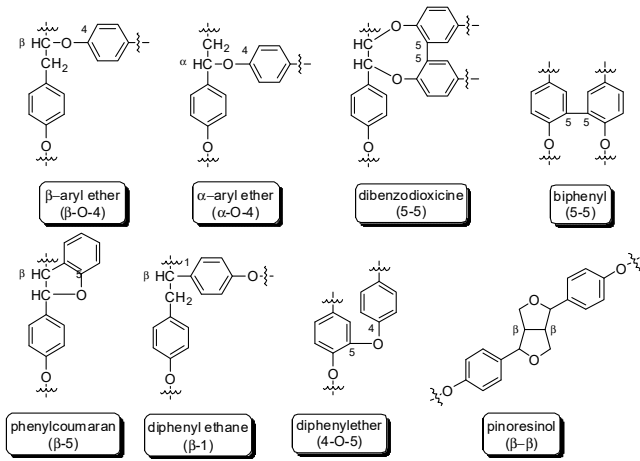


Figure 4. a. Lignin linkages: ether bonds, carbon-carbon bonds, and further linkages.

In 2018, Gou *et al.* reported a detailed study on lignin biosynthesis systematically discussing the monolignol linkage formation [26]. Thus, during biosynthesis three endoplasmic reticulum-resident cytochrome P450 monooxygenases (C4H, C3'H, F5H) are required to generate the three different monolignol precursors. These three monooxygenases are tightly aligned on the cell membranes. However, they obviously do not directly interact with each other but with two other membrane proteins thereby specifically controlling the connectivity of the phenylpropanoid–monolignols. In addition to the different biosynthesis pathways interfering with the formation of monolignol linkages, the pulping and isolation process and corresponding conditions (i.e. temperature, pressure, solvent, pH) do significantly influence and change the lignin structure [19].

Numerous protocols have been developed to elucidate structural properties and compositional patterns that affect the processing of lignocellulose. In 2015, Lupoi and colleagues comprehensively reviewed lignin structure analysis studies, evaluating advantages and disadvantages as well as limitations of a broad number of analytical methods (i.e. FTIR, UV-Vis, Raman, NMR spectroscopy), mass spectrometry, chromatographic methods including SEC, GPC, HPLC, transmission and scanning electron microscopy, thermal analysis via DSC or TGA, as well as X-ray, neutron, and light scattering techniques [27]. The influence of various fractionation techniques (i.e. catalytic oxidative and reductive methods) on lignin structure and morphology is

currently focused in numerous studies [28, 29]. Thus, HSQC spectra of aromatic (dC/dH 100–135/5.5–8.5) and aliphatic (dC/dH 50–90/2.5–6.0) regions of lignin sample were discussed in detail by Chen and Vasilyev [30, 31]. Analogue to these studies, we used the HSQC NMR spectroscopy to study differences in lignin structure obtained from various *Miscanthus X giganteus* genotypes. In addition, we also compared lignins isolated from stem versus leaves (Figure 5) [21].

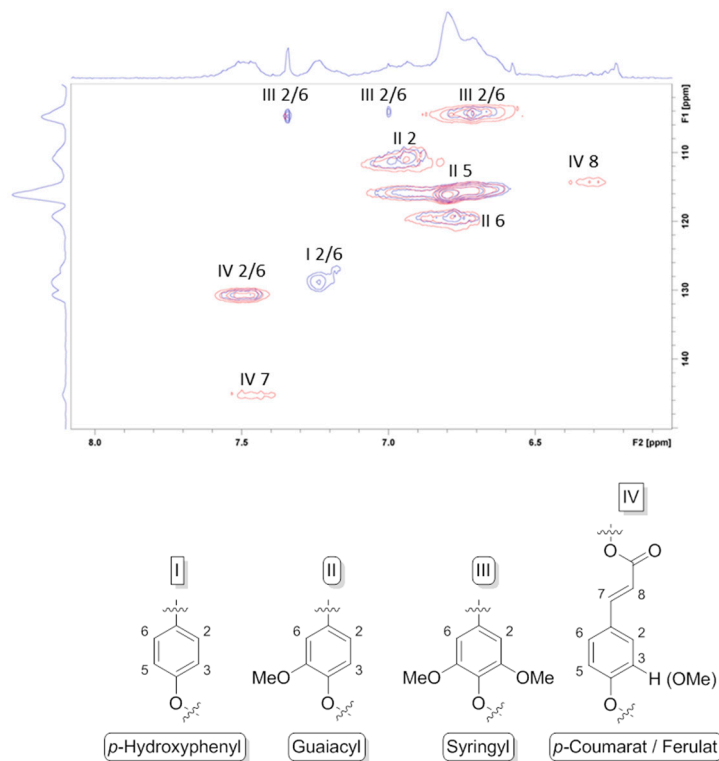


Figure 5. HSQC NMR spectrum, aromatic region (dC/dH 100–150/6.0–8.0) of lignin samples obtained via Organosolv process from *Miscanthus X giganteus*. Comparison of leaf lignin (blue) and stem lignin (red) and corresponding assigned lignin fragments. Copyright Springer 2018.

Cheng and co-worker reported a study combining small angle neutron scattering (SANS) and nuclear magnetic resonance analyses that enabled the description of detailed lignin structure in solution at a molecular level. They performed lignin solubilization studies (in DMSO-d₆ and diluted aqueous NaOD) to investigate correlations between functional groups and resulting ability to form aggregates via intermolecular interactions. Three lignins were investigated: two Kraft lignins (poplar wood, corn cob) and one soda lignin. Intermolecular hydrogen bonding, non-covalent π – π interactions between phenyl rings, lignin chain conformation and the degree of branching were discussed considering operating forces for lignin solubilization [32].

The authenticity of natural products and biomass-derived polymers, such as lignin concerns a number of different characteristics: quantitative and qualitative composition, geographical origin, type of raw material, producer, etc. Today, analytical platforms are developed to holistically prove the authenticity of natural products isolated from animal and/or plant raw materials. Thus, modern analytical methods are currently combined with multivariate data processing. Chemometric modeling of 2D NMR spectra (i.e. DOSY, HSQC, HMBC) are reported using principal component analysis (PCA), independent component analysis (ICA), multivariate regression (PLS), and various discriminant analysis methods (i.e. LDA, FDA, PLS-DA). Quantitative characteristics (molecular weight, content of active ingredients and impurities, pharmacological activity, etc.) and qualitative properties (plant origin, genotype, phenotype, manufacturer) can be determined based on spectrometric and chromatographic profiles as we could recently show for heparins of different origin using 2D NMR and SEC data [33]. This

approach is universal and can be applied to other methods and products as well. Empirical techniques have evolved into statistical approaches (i.e. FTIR, NMR). Sluiter and Krasznai comprehensively reviewed the studies published so far regarding compositional analysis of lignocellulosic biomass and corresponding isolated compounds including lignin [34, 35]. Besides FTIR, Raman, and NMR spectroscopy, neutron and X-ray scattering are appropriate methods to deliver the required data quantity and quality to be used for compositional analysis of biomass-derived compounds including lignin [36]. Various ball-milled *Miscanthus X giganteus* phenotypes were analyzed by Haffner *et al.* using near infrared spectroscopy. PLS regression analysis was used to predict plant extract components such as glucan, xylan, arabinan, acetyl, Klason lignin, total ash, and ash after extraction. Milling to uniform sizes is required since particle size significantly influences the reproducibility of the data [37]. In another study, Hayes *et al.* reported compositional analysis using NIR and UV-Vis spectroscopy of *Miscanthus*. In particular, *Miscanthus* particle size and moisture content were varied using different pre-treatment methods (wet-chopping, air-drying, grounding, sieving). Determined data include glucose, xylose, and Klason lignin [38]. The same spectroscopic methods (NIR, UV-Vis) were applied by Everard *et al.* to estimate the gross calorific value of ground *Miscanthus* and two coppice willow stem samples [39]. Sugarcane lignocellulose was analyzed using diffuse reflectance near-infrared spectroscopy and multivariate calibration by Chong *et al.* in order to determine ash, lignin, and carbohydrate composition data [40].

3. Lignin Antioxidant Capacity and Bioactivity

3.1. Lignin Antioxidant Capacity

Due to their polyphenolic structure, lignins possess antioxidant activity. Kraft lignin from wood sources in pulp industry was reported to be as efficient as vitamin E to protect the oxidation of corn oil [41]. Most antioxidant effects of lignins are considered as derived from the scavenging action of their phenolic structures on oxygen containing reactive free radicals. Although there are several options to study antioxidant activities of naturally occurring phenolic compounds, the DPPH method using 1,1-diphenyl- 2-picrylhydrazyl as a reactive free radical, is recognized as appropriate for lignin structures, analogue to radical scavenging ability of flavonoid and catechin structures. The reactivity of DPPH is far lower than that of oxygen containing free radicals (OH, RO, ROO and O₂), and unlike them the interaction rate is not diffusion-controlled. Dizhbite *et al.* compared DPPH and ABTS (2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) methods and found rather good conformity [42]. As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity [43]. The total phenolic levels can be determined based on their chemical reducing capacity relative to gallic acid or using the Folin–Ciocalteu reagent [44, 45]. Son and Lewis observed DPPH inhibition effects for methylated lignin derivatives [46]. Barapatre and colleagues in detail studied activity differences of aliphatic and free phenolic hydroxyl groups confirming that the radical scavenging activity of phenolic compounds depends on the hydrogen abstraction rate [47]. In our studies we could confirm the proposed mechanism and improve the antioxidant activity of Kraft lignin extracts up to 68 % compared to 55 % for literature values. In addition, the Kraft lignins were compared to Organosolv lignins obtained from beech wood and grasses [21].

3.2. Lignin Antimicrobial Activity

The literature describing the microbial properties of lignins has grown rapidly in the last decade, comprehensively reviewed by Espinoza-Acosta *et al.* [48]. In addition to their effects on antioxidant activity, phenolic hydroxyl and methoxy groups have been reported to be biologically active. Thus, numerous investigations have suggested that lignins can be applied to stabilize food and feedstuffs due to their antioxidant, antifungal, and antiparasitic properties [49]. Commodity products with antioxidant or antimicrobial properties, such as sunscreen

lotions, biocomposites, and clothes that use lignin as a natural ingredient have been prepared, and their characterization has shown promising results [11]. Dumitriu and Popa confirmed in their studies that the main determining factor of the antimicrobial effect of lignin correlates with phenolic fragments and the nature of further functional groups as well as specific side chain constitution. Typically, the presence of a double bond in α , β positions of the side chain and a methyl group in the γ position grants the phenolic fragments the most potency against microorganisms [50].

Primary antimicrobial study performed with Kraft lignin extracts showed that purification strongly influence the lignin bioactivity against *S. aureus* and *L. monocytogenes* (gram positive bacteria) and *E.coli* (gram negative bacteria) [21]. Lignin nanoparticles incorporated in polylactic acid (PLA) revealed an innovative capacity to inhibit the bacterial growth along the time [49]. The decrease of oxidative and inflammatory damage to the kidney in streptozotocin-induced diabetic rats due to lignin-derived lignophenols was reported by Sato *et al.* [51]. Similar to these results, low molecular weight lignins were tested regarding their potential as anti-emphysema agents *in vitro* [52].

Additionally, other properties such as anticarcinogenic, apoptosis-inducing antibiotic, and anti-HIV activities have been reported for lignin-carbohydrate complexes (LCCs). The toxicity of free radicals contributes to cellular damages like DNA and protein damages, inflammation processes, tissue injury and cellular apoptosis which could cause cancer development. Barapatre *et al.* showed an antioxidant and antidiabetic efficiency of modified alkali lignin. The antidiabetic property has been investigated in terms of *in vivo* glucose movement inhibition and α -amylase inhibition. The modified samples effected the α -amylase inhibition and an increased glucose binding efficiency evaluated by decreased glucose diffusion [53]. Studies of Hasegawa *et al.* were focused on the evaluation of lignosulfonic acids in relation to α -glucosidase activity. Their results suggest a suppression of blood glucose via inhibition of the α -glucosidase and intestinal glucose absorption. Here, lignosulfonic acid is a reversible and non-competitive inhibitor [54]. Besides antioxidant and antidiabetic properties lignins seem to have influence on the secretion of apolipoprotein B and cholesterol levels and thus play a role in obesity control. This was investigated by Norikura presenting lignophenols to a human hepatocellular carcinoma cell line leading to reduced levels of apo-B and cholesterol [55].

Furthermore, lignins are also studied regarding their antiviral capacity. Gordts *et al.* investigated lignosulfonic acid regarding HIV antiviral activity revealing lignosulfonic acid to be a potent inhibitor of the HIV replication. They also prevent an uptake of the virus by CD4T cells from persistently infected T cells *in vitro* [56]. The antiviral activity of lignin products is also shown for CMV and HSV- 1 and HSV- 2. Ligno-carbohydrates seem to inhibit the viral binding, penetration and replication [57, 58]. The inhibition of the replication of herpes simplex virus (HSV) was studied by Andrei and colleagues [59]. They found, that topical tenofovir, a microbicide effective against HIV, inhibits herpes simplex virus-2 replication.

Henry and colleagues reported that lignins do also show anticoagulant effects. In particular, they investigated low molecular weight lignins regarding their inhibiting influence on thrombin and factor Xa through allosteric disruption of the enzymatic apparatus [60]. In accordance to these results, Mehta *et al.* published similar studies focusing sulfated β -O4 lignins, which act as an allosteric inhibitor of thrombin to reduce fibrinogen cleavage resulting in a reduction of platelet activation [61].

4. Lignin-Derived Biomaterials for Drug Encapsulation/Release and Tissue Engineering

4.1. Gels and Hydrogels for Drug Encapsulation and Release

Considerable interdisciplinary research efforts have been focused on the design of biomaterials for drug delivery applications. However, kinetically controlled release still remains a challenge due to a number of open questions regarding the chemical and biological criteria that limit drug delivery. Within the last fifty years, a broad variety of encapsulation and

release materials have been designed to release bioactive drugs for an extended time period and (in best case) initiate specific interaction with the host in order to control the released drug amount. Basic release mechanisms include: matrix tortuosity-controlled diffusion, membrane controlled diffusion for small molecules and hydrogels via mesh size and network swelling (Figure 6) [62].

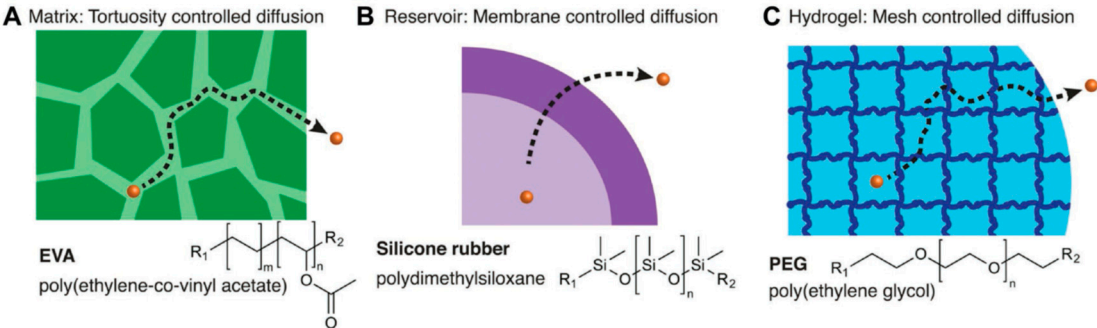


Figure 6. Examples of controlled release platforms. A. Matrix tortuosity-controlled diffusion; B. Membrane controlled diffusion; C. Hydrogels. [62] Copyright 2018 John Wiley and Sons.

In the past decade, the number of research groups working on “lignin valorization” steadily increased, including lignin-based gels and hydrogels for controlled and/or sustained release of pharmaceutical drugs and compounds such as pesticides used in agriculture. In 2015, Velez and colleagues first reported the development of antimicrobial nanoparticles with biodegradable cores, prepared from Indulin AT lignin loaded with silver cations and coated with a cationic polyelectrolyte (polydiallyldimethylammonium chloride, PDAC) (Figure 7). The lignin-derived nanoparticles showed biocidal activity on both Gram-negative and Gram-positive human pathogens as well as quaternary amine-resistant bacteria at significantly lower silver concentrations compared to conventional reagents [63].

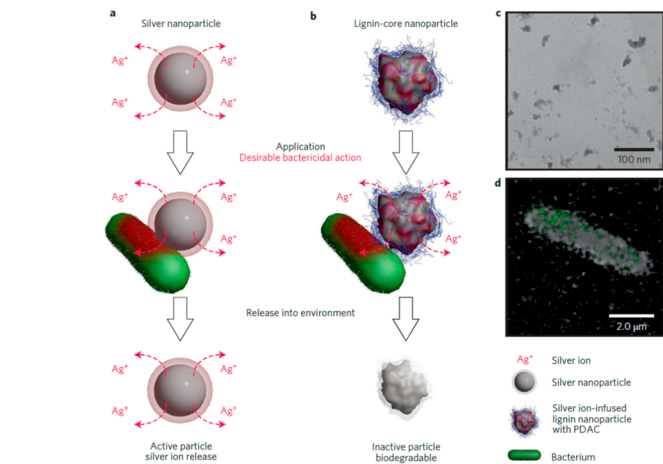


Figure 7 Schematics of the general use cycle and principle of the bactericidal action of environmentally benign lignin-core nanoparticles (EbNPs) and the currently used silver nanoparticles (AgNPs). **a**, General mechanism for the antimicrobial action of common AgNPs via the release of Ag⁺ ions, which continues post-utilization. **b**, Mechanism of antimicrobial action of Ag⁺ ion-infused EbNPs with a cationic polyelectrolyte coating that facilitates electrostatic attraction between the EbNPs and negatively charged cell walls. In contrast to AgNPs, EbNPs are depleted of Ag⁺ ions during their application, minimizing their post-utilization activity. **c**, TEM micrograph of as-synthesized EbNPs in the size range of 40–70 nm. **d**, Confocal microscopy image of EbNPs with polyelectrolyte coating adhering to the cell membrane of *E. coli*.

Figure 7 An environmentally benign antimicrobial nanoparticle based on a silver-infused lignin core. [63] Copyright 2018 Springer Nature.

In June 2018, Österberg and colleagues reported another “breakthrough in lignin research”: the synthesis of colloidal cationic lignin nanoparticles to encapsulate enzymatic biocatalysts to be used for esterification in aqueous media. The catalyst is immobilized (“spatially confined”) within the lignin-derived colloidal particles [64]. Recent approaches in the development of lignin-derived nanoparticles generated for biomedical applications were reviewed by Beisl and

369 Shi [65, 66]. In particular, nano-structured lignin hydrogels, their synthesis, characterization and
370 possible applications were reported by Thakur and Kai [67, 68]. Table 3 summarizes
371 lignin-derived encapsulation materials (macro- and nanosized derivatives) studied regarding
372 their bioactivity and release performance.

373 **Table 3.** Lignin-derived systems for biomedical applications: drug release, antioxidant and antibacterial use
374 and scaffolding.

Application	Matrix Type	Encapsulation Method and Active Ingredient	Results	References
drug release	lignin nanoparticles from Indulin AT	nanoparticle flash precipitation with subsequent silver ion infusion and polyelectrolyte coating	> 95 % release of silver ions in 24 h and anti-bacterial effect against <i>E.coli</i> , <i>P.aeruginosa</i> and <i>Rastonia sp.</i>	Richter <i>et al.</i> 2015 [63]
drug release	lignin nanoparticles from LignoBoost™ softwood Kraft lignin	incorporation of poorly water-soluble Sorafenib® and Benzazulene® during particle formation via polarity change	poorly water-soluble drugs are released upon degradation of the particles; the water-soluble drug could not be incorporated into the NP; low cytotoxic effects on cancer cell lines: MDA-MB-231, MCF-7, PC3-MM2, Caco-2 and non-tumor cells: KG1 and EA.hy926 endothelial cells	Figueiredo <i>et al.</i> 2017 [72]
drug release	lignin nanospheres from enzymatic hydrolysis lignin	no drug loading	lignin nanoparticles with tunable size can be produced via self-assembly	Xiong <i>et al.</i> 2017 [73]
drug release	lignin nanoparticles from alkaline lignin	incorporation of Resveratrol® during particle formation via polarity change	about 80 % drug released into PBS after 4 days	Dai <i>et al.</i> 2017 [74]
drug release	polyelectrolyte microparticles of quaternary ammonium lignin – sodium dodecyl benzenesulfonate (lignin from pine alkali lignin)	loading of hydrophobic Avermectine during particle precipitation	release of ~80 % Avermectine into methanol:water (1:1) after 72 h; good UV-protection of the drug (85 % preserved after 96 h UV irradiation 30 W, 310 nm)	Li <i>et al.</i> 2018 [75]

drug release	lignin droplets in W/O Pickering emulsion coated with polyurea	loading of hydrophobic Avermectine in emulsion before droplet coating reaction	release of 85 % of Avermectine into 4:1 ethanol:water after 72 h; lignin-polyurea coatings were more porous than pure polyuria layers, which showed a more sustained release; UV-protection of lignin coatings was good (>75 % preserved after 120 h irradiation 30 W, 310 nm)	Pang <i>et al.</i> 2018 [76]
drug release	montmorillonite/ lignin-acrylamid e-isopropyl acrylamide copolymer	adsorption of methylene blue from aqueous solution	effective removal of dyes from aqueous solutions over multiple sorption/desorption cycles	Wang <i>et al.</i> 2017 [77]
drug release	crosslinked cellulose-lignin hydrogels (steam expansion lignin, aspen wood)	swelling of gel in polyphenol solution	a higher lignin content leads to a faster drug release, up to 30 % in 10 h	Ciolacu <i>et al.</i> 2012 [78]
antibacterial effect	lignin nanoparticles in polyethylene films (Björkman lignin from beech wood flour)	none	lignin particles exhibit antibacterial effect against <i>E. coli</i> and <i>S. aureus</i> in the same order of magnitude as other antibacterial agents such as Bronopol and chlorohexidine	Gregorova <i>et al.</i> 2011 [80]

375
376 Thus, lignin is shown to be a promising resource for biodegradable bioactive materials,
377 either for agricultural formulation of e.g. pesticides or fertilizers studied by Chowdury or in
378 colloidal form as “green” alternative for metallic nanoparticles in pharmaceutical drug delivery
379 [69, 70]. Lignin colloidal spheres are also tested as sunscreen additives in cosmetics. Qian *et al.*
380 produced sun screen lotions with lignin spheres of about 50 nm that reached a sun protection
381 factor (SPF) of about 15 for UVA radiation. However, the SPF seems to be dependent on both size
382 and extraction method of the lignin spheres [71]. Figueiredo *et al.* developed lignin nanoparticles
383 via self-assembly during dialysis. They incorporated iron or iron oxide into the lignin particles to
384 get magnetic particles and performed drug release studies of the poorly water-soluble drugs
385 Sorafenib® and Benzazulene® on pure lignin particles. All variations of particles showed low
386 cytotoxicity. Drug release kinetics of hydrophobic drugs depends on the pH of the release
387 medium, because of accelerated lignin particle degradation in more alkaline media: at both pH
388 5.5 and 7.4 (aqueous buffer with 10 % fetal bovine serum) more than 90 % of the drug was
389 released in the first 6 hours. However, in pure water, the drug could be retained in the particles
390 for 15 days [72]. Nanospheres of enzymatic hydrolysis lignin were prepared by Xiong *et al.* via
391 self-assembly by adding a non-solvent to the lignin solution. The group was able to prepare

spheres in the range of 190-590 nm with a good stability over 30 days of storage. However, albeit proposing a possible carrier function, no experiments regarding drug release or biocompatibility have been performed in this study [73]. In 2017, Dai *et al.* reported the synthesis of lignin nanoparticles as a green carrier for the sustained Resveratrol® drug release. The group encapsulated the hydrophobic drug Resveratrol® together with Fe₃O₄ nanoparticles for possible use in targeted cancer therapy. The particles showed a sustained drug release of 80 % over 4 days. Both, *in vitro* and *in vivo* biocompatibility and anticancer tests of particles with and without magnetic particles revealed no adverse effects on cells or mice [74]. Just recently, Li *et al.* prepared polyelectrolytic microparticles from quarternary ammonium lignin and sodium dodecyl benzenesulfonate via particle precipitation. They loaded the particles with hydrophobic insecticide Avermectine during the precipitation step and investigated both drug release and UV protection capability of the microparticles. The release into 1:1 methanol:water was somewhat sustained with about 80 % of the drug being released in 72 h. The anti-photolysis properties of the lignin proved to be very good, after 96 h of UV irradiation 85 % of the drug was still preserved in the spheres [75]. The same group also reported lignin droplets in a Pickering emulsion coated with polyurea for a sustained Avermectine release. Here, the drug was loaded to the droplet during emulsion before the coating step. Polyurea-coated lignin spheres proved to sustain the Avermectine release into 4:1 ethanol:water less than pure polyurea coatings (85 % in 72 h and 50 % in 72 h, respectively). The authors found that the lignin-polyurea layer is much more porous than the pure polyurea layer due to the 3D structure of the lignin. However, UV-protection of the microparticles proved to be very good, after 120 h of irradiation, more than 75 % of the drug was preserved [76].

In addition to nanospheres, Wang *et al.* prepared a hybrid hydrogel of montmorillonite and a lignin-derived graft copolymer (lignin-g-acrylamide-isopropyl acrylamide) that could be used as an effective agent for the removal of dyes or other chemicals from aqueous solutions. At room temperature and neutral pH the hybrid hydrogel outperformed other hydrogel systems in the adsorption of methylene blue by a factor of 4 – 10 [77]. Further examples for lignin-based composites are cellulose-lignin hybrid hydrogels, which were prepared by Ciolacu *et al.* in 2012. Here, cellulose and lignin were crosslinked with epichlorohydrin resulting in porous materials after lyophilization. Drug release from those gels was investigated using different polyphenols as model drugs. Loading was performed by swelling the dried gels in polyphenol solution. Both swelling and subsequent drug release is dependent on lignin content in the gel. Higher lignin ratios lead to both a higher swelling and a faster and higher release of polyphenols. However, drug release has only been monitored for about 10 h, resulting in a maximum of 30 % of the released drug [78].

Besides spheres and gels, lignin-based films were synthesized and tested for biomedical applications. Kosikova *et al.* reported thin films of a lignin-polypropylene blend to have improved antioxidant properties against thermos-oxidative degradation. Furthermore, the use of lignin as stabilizer in plastics as positive effects on the protection of mice DNA against oxidation damage due to lignin's scavenging effects [79]. Gregorova *et al.* also prepared thin films, using polyethylene and lignin nanoparticles. The lignin acts as antibacterial agent and its effectiveness against *E. coli* and *S. aureus* was found to be equal to other bactericides such as bronopol or chlorohexidine. Moreover, the addition of the lignin did not alter the mechanical properties of the films [80].

4.2. Lignin-based Scaffolds for Tissue Engineering

Research on biomaterials for tissue engineering and regenerative medicine covers various interdisciplinary aspects: depending on the final application, scaffolds have to fulfill a number of very different, sometimes even contradictory requirements. So, bone regeneration scaffolds are required to show sufficient mechanical stability when implanted combined with controlled degradability to be replaced by natural bone thereby avoiding toxic degradation products. So far, bone replacement materials include: autologous transplants (source: chin area, retro-molar

region, iliac crest, trabecular bone), allogeneic transplants (availability via bone banking), xenogeneic transplants (temperature or chemical pre-treatment), alloplastic (hydroxyapatite, tri-calcium phosphate, ceramics, polymers on the basis of α -hydroxyl acid). Novel stem cell based approaches allow individualized patient-specific solutions. Biomaterials are specified by: biocompatibility according to ISO standards including long-term studies, stability against physiological media (pH, temperature), mechanical stability depending on specific application (e.g. stress/strain, elongation, impact moduli etc.), corrosion resistance (for metallic components), residual-free metabolism in case of biodegradable materials, and appropriate technical functionality according to specific application. First step in cell-scaffold interaction are cell adhesion processes related to intensive interaction on cell – biomaterial surface and interfaces. These interactions strongly depend on surface polarity (hydrophilic versus hydrophobic surfaces), surface roughness and topography. The scaffold development starts with polymer synthesis using state-of-the-art polymerization techniques to achieve well-defined porous structures to enable cell ingrowth. Polymer bulk and surface have to be tailored to meet needs of the natural environment. Scaffold surface polarity and topography have to be adapted to the cell shape in order to support cell adhesion, proliferation and growth [81, 82].

In the following, the focus is drawn onto stem cell-based approaches in bone regeneration using scaffold materials that in detail influence the differentiation and proliferation of mesenchymal stem cells (MSC). They are found in all adult mesenchymal tissues and play a role in the maintenance of tissue homeostasis and repair by allowing renewal of the cellular stock. MSCs can be isolated from both human and animal sources. Adipose tissue is a rich and promising source of these cells. Adipose-derived stem cells (ASCs) are often effective and safe, and have been used in preclinical and clinical studies for both autologous and allogeneic transplantation. The potential use of stem cell-based therapies for the repair and regeneration of various tissues and organs provides an important alternative therapeutic solution for the treatment of many diseases [83-86]. MSCs have the potential to differentiate into multiple mesenchymal derived lineages (Figure 8).

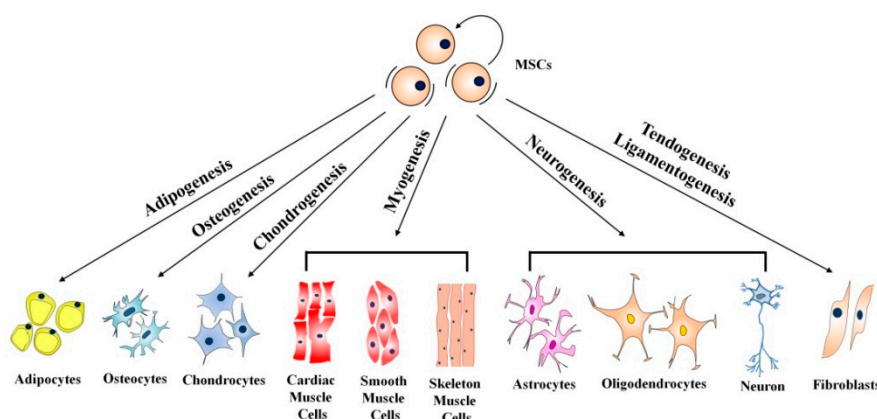


Figure 8. The differentiation potential of mesenchymal stem cells [13]. Copyright 2018 Springer eBook.

Adipogenesis leads to adipocytes and osteogenesis to osteocytes. Similar, chondrogenesis will end with chondrocytes. Myogenesis will generate cardiac, smooth, and skeletal muscle cells and neurogenesis will lead to astrocytes, oligodendrocytes, and neurons. After tendogenesis and ligamentogenesis fibroblasts are produced. MSCs can be isolated from different body parts for instance; with amniotic fluid, umbilical cord, dental tissue, bone marrow, peripheral blood, skin, and adipose tissue being the most common sources. Following the isolation process, the MSCs can be differentiated towards osteoblasts using a suitable differentiated medium. Appropriate scaffolds seeded with osteoblasts is considered to become the best choice for future bone regeneration.

Stem cells are affected by their microenvironment which is defined by extracellular matrix properties such as elasticity and geometry, molecules which connect to the extracellular matrix for instance transforming growth factor- β (TGF- β), tension induced proteins (TIPs), integrins and transient receptor potential (TRP) which can regulate cytoskeleton tension successively followed by gene expression and focal adhesion through the activation of a series of mechanical transduction events. Also various soluble factors such as extracellular nucleotide, growth factors and cytokines influence stem cell fate. Mechanical forces such as shear stress and blood pressure influence stem cell proliferation and differentiation from the media side of the niche as well as chemical and physical factors like pH or oxygen (Figure 9).

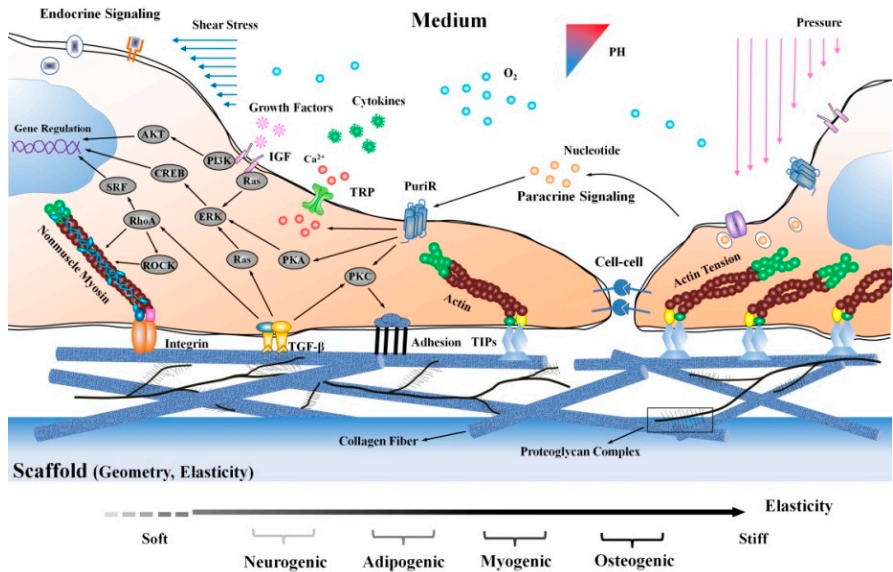


Figure 9. Stem cell and their natural microenvironment. Factors influencing the stem cell niche can be roughly categorized in three groups: physical and mechanical factors like (a) shear forces, elasticity and topography, (b) cellular issues like immune and nerve cells, nearby blood vessels and neighboring stem cells and (c) soluble factors such as oxygen, glucose, hormones, growth factors or signaling molecules [13]. Copyright 2018 Springer Verlag.

Delella and her colleagues reviewed the state-of-the-art regarding the control of mesenchymal stem cell manipulation process prior to their use. In particular, they studied the effect of the endocrine disruptor bisphenol A (BPA) on MSC fate and tried to explain the mechanisms by which BPA interferes with adipogenesis and increases adipose tissue in humans [87]. Since lignin-derived compounds are also discussed to become a BPA substitute, their potential effect on MSC fate has to be investigated more in detail [88].

So far, a large variety of materials are studied regarding their ability to support MSC differentiation, proliferation and growth, both *in vitro* and *in vivo*: natural polymers (i.e. gelatin, alginate, silk, and collagen), synthetic polymers, glasses and ceramics or HA-based composites and hybrids prepared via conventional techniques or as nanostructured materials via additive manufacturing [89]. Nanofabrication techniques for scaffold generation include rapid prototyping (RP) methods such as selective laser sintering (SLS), selective laser ablation (SLA), fused deposition modeling (FDM), chemical and physical vapor deposition (CVD, PVD), 3D printing methods resulting in tailor-made layered, cubic and spherical structures (Figure 10).

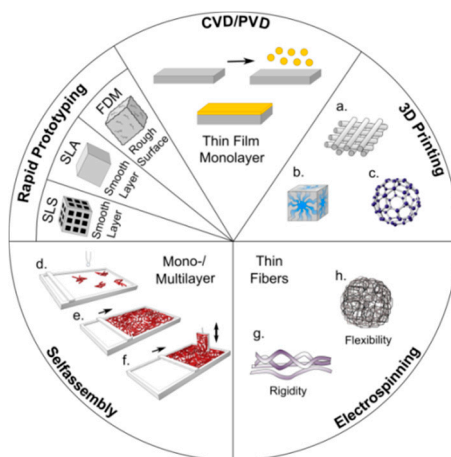


Figure 10. Fabrication methods used for the development of nanostructured scaffolds for tissue engineering applications: 3D printing, electrospinning, rapid prototyping and self-assembly techniques such as Langmuir-Blodgett.

In addition, self-assembly methods such as Langmuir-Blodgett technique for monolayer formation and electrospinning are used for scaffold fabrication aiming bone and vascular tissue [15]. As already discussed for the hydrogel production, lignin-derived nanoparticles (spheres, rods, films) are prepared for various applications, such as encapsulation of drugs and dyes or nanofiber production [64-67]. Xu and colleagues studied and reviewed the potential of various lignins for 3D printing in very detail including various processing parameters [90].

Among the large number of materials reported so far for stem-cell-based tissue engineering, only a very few developed from lignocellulose feedstock and/or lignin derivatives. First, Rekola *et al.* in 2009 reported the osteoconductivity of heat-treated wood bone implants. Without investigating lignin in particular the group found that a heat treatment of wood increased the biological behavior of such implants, a higher temperature resulting in improved *in vitro* osteoconductivity [91]. An alginate-lignin composite aerogel has been prepared by Quraishi *et al.* The group mixed solutions of alginate and lignin and used CO₂ induced gelation and foaming to produce aerogels with μm-sized interconnecting pores. Aerogels showed low stiffness in the range of fibrous tissue but no cytotoxic effects on mouse fibroblast-like L929 cells *in vitro* [92]. Farhat *et al.* produced a variety of different polysaccharide-based composites using a reactive extrusion process. Starch, hemicellulose or lignin were cross-linked with citric acid and the corresponding hydrogels were characterized by means of swelling, mechanical strength and degradability. Swelling is depending on pH but also on the amount of citric acid used as cross-linker. Degradation rates were studied at physiological condition for 15 days. Degradation could be reduced using additional catalysts during polymer extrusion. Dynamic mechanical analysis revealed that the hydrogel degradation induces significant reduction in the compressive modulus [93, 94]. Agarose-lignin hydrogels were prepared and studied regarding their mechanical behavior by Techato and colleagues. Agarose solubilized in water forms a gel with a rigid network, resulting on a three-dimensional porous structure. Furthermore, agarose hydrogels may be polymerized *in situ* thereby allowing the hydrogel to acquire the required shape. Ligin obtained from oil palm empty fruit bunches is used to generate lignin-agarose hydrogel with epichlorohydrin as the cross-linking agent. The gel strength of composite lignin-agarose hydrogel was studied by texture analysis [95]. Very recently, Morganti discussed the potential of chitin and lignin to be used as natural scaffold materials imitating the extracellular matrix (ECM). The authors prepared composites consisting of nanoscaled lignin and chitin nano-fibrils of high surface area-to-weight ratios [96]. Wang *et al.* reported reinforced chitosan microfibers prepared by adding various amounts of lignin during the spinning process. They showed that addition of 3-5 % lignin improves tensile strength and stiffness of chitosan. The authors predict good biocompatibility without proving this by experimental data [97]. In 2015, Anwer *et al.* prepared various poly (lactic acid) (PLA) – lignin composites and studied their

mechanical properties. They found that a lignin filler content of up to 15 % negatively influences the tensile strength of the composites, and that the crystallization of PLA is also slowed down [98]. Just recently, Spiridon and Tanase also published a study on PLA-lignin composites. Here, addition of up to 7 % lignin microparticles led to a decrease in tensile strength, however, adding 7 – 15% lignin increased the tensile strength. Additionally, biocompatibility of the composites was tested on SaOS-2 cells with no observed adverse effects [99]. Lignin-based copolymers comprised of lignin-poly(ε-caprolactone-co-lactide) were synthesized via solvent-free ring-opening polymerization and subsequently spun into blend nanofibers. The copolymers were blended with either polycaprolactone (PCL) or poly (L-lactic acid) (PLLA) during electrospinning. The spun mats were evaluated regarding their mechanical properties, antioxidant activity and biocompatibility. The PCL-blends were mechanically improved; however, the stability of the PLLA-blends was slightly decreased. Antioxidant activity and biocompatibility on the other hand were improved. PLLA-blends showed an increased viability and proliferation of NIH/3T3 fibroblasts, making them interesting candidates for tissue engineering applications [100]. Erakovic *et al.* prepared a bioactive coating for titanium implants. The coatings comprised of hydroxyapatite (HA) and Organosolv lignin in various ratios were deposited onto the implants electrophoretically and were sintered afterwards. The coatings showed good biocompatibility, and, when doped with silver during deposition, even a good antibacterial effect against *S. aureus* [101]. Table 4 summarizes examples of lignin-derived materials reported to be candidates for scaffold utilization.

Table 4. Lignin-derived scaffold for possible bone tissue engineering applications.

Aim	Matrix Type	Additional Ingredients	Results	References
osteoconductivity	heat-treated birch wood	none	heat-treatment of wood increases osteoconductivity	Rekola <i>et al.</i> 2009 [91]
scaffold fabrication	alginate-lignin aerogel (lignin from wheat straw by enzymatic hydrolysis)	none	fluid uptake in Tris-HCl buffer of > 1600 %, good biocompatibility	Quraishi <i>et al.</i> 2015 [92]
scaffold fabrication	starch, lignin (from kraft lignin) or hemicellulose	none	hydrogels produced by reactive extrusion show pH dependent swelling behavior (water uptake at pH 9: from 400 to 1400 %); the amount of citric acid used as cross-linker also influences both swelling and degradation of the hydrogels. Additional catalysts used during extrusion slow down degradation.	Farhat <i>et al.</i> 2017 [94]
scaffold fabrication	agarose-lignin composites (lignin from Kraft black liquor)	none	crosslinked agarose-lignin hydrogels exhibit enhanced mechanical properties compared to pure agarose	Techato <i>et al.</i> 2018 [95]

				gels
influencing mechanical properties	lignin-chitosan microfibers	none	improving mechanical properties of chitosan fibers by adding 3 % lignin	Wang <i>et al.</i> 2016 [97]
influencing mechanical properties	poly(lactic acid) with lignin as filler (Kraft lignin)	none	lignin as filler does not decrease storage modulus, but inhibits PLA crystallization	Anwer <i>et al.</i> 2015 [98]
influencing mechanical properties	poly(lactic acid) with up to 15% lignin as filler (Organosolv lignin from birch wood and Kraft lignin from softwood)	none	higher lignin content leads to higher tensile strength, but also slightly decreased water sorption capacity. Organosolv lignin yields slightly better mechanical results; good biocompatibility against SaOS-2 cells regardless of lignin type.	Tanase <i>et al.</i> 2018 [99]
influencing mechanical properties	lignin-based copolymer / polyester blend nanofibers (alkali lignin)	none	mechanical improvement dependent on polyester, good antioxidant activity and biocompatibility against NIH/3T3 fibroblasts	Kai <i>et al.</i> 2017 [100]
bioactive coating for implants	hydroxyapatite/lignin composite coatings on titanium (Organosolv lignin)	doping of silver for antimicrobial effect	HA coatings on Ti were non-cytotoxic to peripheral blood mononuclear cells; Ag-doped coatings showed antibacterial behavior against <i>S. aureus</i>	Erakovic <i>et al.</i> 2014 [101]

There are also a number of studies on polyurethanes (PU) used in tissue engineering. Due to the broad variety of available (stiff or flexible) polyol and isocyanate components for polyurethane synthesis, their internal structure and morphology can be tuned to resemble natural bone and promote tissue ingrowth [102, 103]. Biodegradable water-based shape memory polyurethane scaffolds for bone regeneration were prepared by Wang and colleagues using 3D printing (via low-temperature fuse deposition). Supramagnetic iron oxide nanoparticles were incorporated to promote osteogenic induction. Scaffolds seeded with hBMSCs showed improved osteogenesis compared to conventional PU scaffolds [104]. Since lignin-based polyurethanes are already available in various compositions with tunable mechanical stability and degradability, lignin-PU in future will become favored candidates to be studied in detail regarding their ability to be used for both, biodegradable tissue engineering scaffolds and drug encapsulation materials [105, 106].

Within the last five years, calcium phosphate cements and hydroxyapatite-derived hybrid materials gained increasing interest since they are shown to combine scaffold function with additional ability for sustained release, in detail studied for a number of different drugs including low molecular weight (i.e. antibiotics, anticancer drugs) and high molecular weight compounds (i.e. proteins, growth factors) and ions (i.e. Ca, Sr, Si, Zn, Mg) [107]. Due to their

osteoconductivity and injectability they are already used as bone grafts. Moreover, their low-temperature setting reaction and intrinsic porosity allow drug incorporation and release. Osteogenesis of MSCs is known to be influenced by a broad number of osteoinductive and osteoconductive compounds, respectively, such as growth factors. Recently, we could show that stem cell differentiation toward bone is guided by various purinergic receptors (P2X and P2Y), Figure 11 [108, 109, 110].

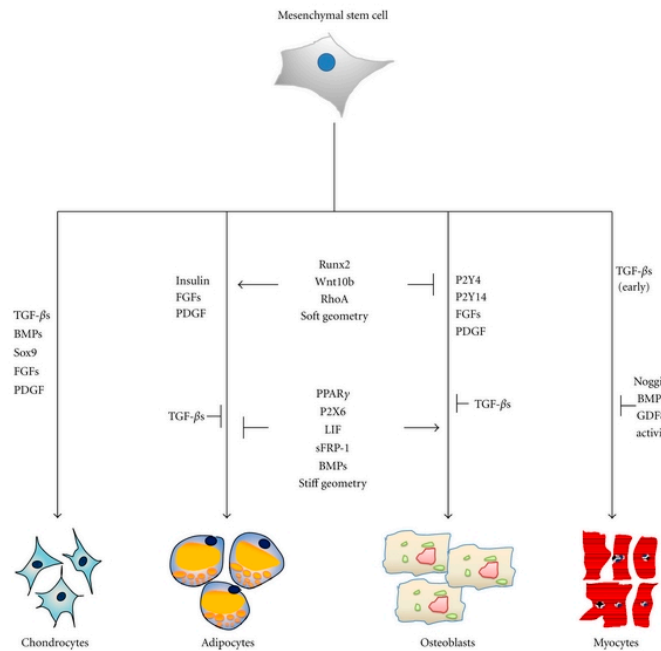


Figure 11: Key molecules regulating adipogenesis and osteogenesis in mesenchymal stem cells. Runx2, Wnt10b, RhoA, and soft geometry can induce osteogenesis while inhibiting adipogenesis. [108]. Copyright 2018 under the Creative Commons Attribute License.

Thus, controlled release of corresponding P2 ligands (agonists, antagonists) encapsulated and kinetically controlled released during MSC differentiation could be used to tailor MSC differentiation into cardiovascular and bone tissue, respectively [110]. First hybrid materials based on HA and polysaccharide (agarose derivatives) have been prepared to be used as appropriate scaffold for MSC differentiation and guided ligand release [111]. Here, lignin could be used in future to partially substitute the polysaccharide components and thereby improve mechanical stability as shown for the agarose/lignin composites.

5. Conclusion and Perspectives

Highly advanced biomaterials are required for stem cell based approaches in tissue engineering. First studies on lignin-derived composites do confirm its potential to be used for scaffold and/or drug release development. Antioxidant and antimicrobial capacity of lignin extracts allow a broad variety of potential applications, such as drug in cancer therapy. Although the isolation of well-defined lignin fractions is still challenging, the intrinsic bioactivity will be the driving force for successful implementation of lignin-derived biomaterials in medicine. Regarding lignin structure analysis, compositional data processing will become more important to specify detailed structural differences due to biomass source, plant genotypes or isolation conditions. Future efforts in scaffold development for bone regeneration most probably are directed toward nanostructured hydroxyapatite-derived hybrid materials imitating the complex natural bone composition. While conventional manufacturing methods are mainly based on chemical induction of differentiation via growth factors and cytokines, concentrating on altering material properties such as substrate stiffness and topography to mimic the stimuli stem cells receive in their natural niche gets more into focus. First promising lignin-derived

nanocomposites are reported, they will be studied in detail regarding their ability for kinetically controlled release to guide stem cell differentiation and finally improve tissue regeneration.

Acknowledgments: Financial support via BMBF program “IngenieurNachwuchs” projects “LignoBau” (03FH013IX4) and “PersoImplant” (03FH019IX5); BRSU Graduate Institute for scholar ship (A.A., M.B., B.E.-H., D.H.) and Erasmus-Mundus Avempace-II scholar ship (A.A.).

Author Contributions: A.A., M.B., B.E.-H., D.H., S.E.K. and M.W. performed the experiments, analyzed the data and contributed writing the manuscript; B.K., J.K. and E.T. contributed materials and analysis tools; M.S. conceived and designed the experimental studies and wrote and formatted the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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