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Ultrasound Driven Assembly of Lignin into Microcapsules for Storage and Delivery of Hydrophobic Molecules

Mariarosaria Tortora,[†,‡] Francesca Cavalieri,[†] Pasquale Mosesso,[§] Flavia Ciaffardini,[§] Federica Melone,[†] and Claudia Crestini*[,†]

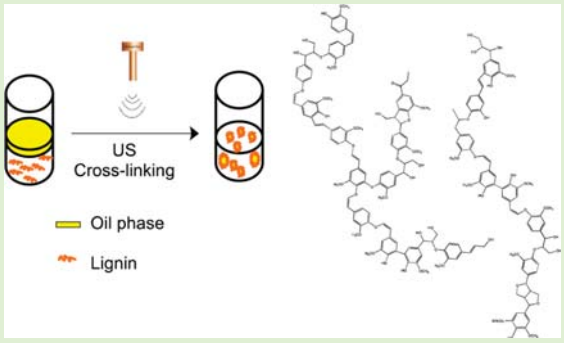
[†]Dipartimento di Scienze e Tecnologie Chimiche, Tor Vergata University, Roma, Italy

[‡]Istituto di Biochimica delle Proteine − IBP, CNR, Napoli, Italy

[§]Dipartimento di Scienze Ecologiche e Biologiche, Università degli Studi della Tuscia, Viterbo, Italy

Supporting Information

ABSTRACT: Oil-filled microcapsules of kraft lignin were synthesized by first creating an oil in water emulsion followed by a high-intensity, ultrasound-assisted cross-linking of lignin at the water/oil interface. The rationale behind our approach is based on promoting documented lignin hydrophobic interactions within the oil phase, followed by locking the resulting spherical microsystems by covalent cross-linking using a high intensity ultrasound treatment. As further evidence in support of our rationale, confocal and optical microscopies demonstrated the uniformly spherical morphology of the created lignin microparticles. The detailed elucidation of the cross-linking processes was carried out using gel permeation chromatography (GPC) and quantitative ^{31}P NMR analyses. The ability of lignin microcapsules to incorporate and release Coumarin-6 was evaluated in detail. In vitro studies and confocal laser scanning microscopy analysis were carried out to assess the internalization of capsules into Chinese hamster ovary (CHO) cells. This part of our work demonstrated that the lignin microcapsules are not cytotoxic and readily incorporated in the CHO cells.



INTRODUCTION

In the recent years, microencapsulation processes have been the subject of significant academic and industrial interest.[1−9] The technique is based on the confinement of solid, liquid, or gaseous materials in micrometer-sized capsule shells. Generally, the capsule's core may contain pesticides, poorly soluble drugs, flavors, and food additives.[10−16] The capsule's shell prevents and controls the diffusion of the confined compounds from the core, providing a barrier of micro- to nanometer thickness. In addition, the shell protects the sensitive compounds from acidic, alkaline or enzymatic degradation environments and ensures target/timed delivery of the encapsulated components. The release rate is affected by the diffusion coefficient of the substance through the capsule's shell, the thickness of the shell, and the mechanical and chemical stability of the microcapsules.[17] Chemical and physic-chemical details related to the material of the shell play a significant role in controlling the diffusion coefficient of the encapsulated substance. For instance, the kinetics of release can be tuned by changing the shell's degree of cross-linking.[18−20] The biocompatibility of the encapsulating matrix is a requirement for cosmetic, pharmaceutical, and food applications. The synthesis of microcapsules can be carried out via a variety of methods. The choice of the method is determined by the nature of the specific core and/or shell materials and the end use of the particles. In order to develop a new microcapsule delivery system based on cross-linked lignin, we decided to use the ultrasonic approach, as it combines two different phenomena: emulsification and cavitation, the latter responsible of the cross-linking reactions.

Lignin is the second most abundant plant biopolymer on earth; it is commonly derived from wood and plays an important function in providing mechanical support to bind plant fibers together. Lignin represents the main byproduct of the pulp and paper manufacturing industry,[21] and it is nowadays emerging as a new inexpensive and renewable starting material for the development of new products and processes. Furthermore, modern biorefinery approaches produce lignin as their main side product. Hence, the exploitation of such a biocompatible and safe material is of crucial relevance for the development of novel, value added biorefinery streams.[22] To our knowledge, lignin-based micro- or nanocapsules have never been reported. The possibility to produce micro- or nanostructured systems based on lignin, which is known to be an antioxidant[23,24] and a UV light[25,26] absorbing biomacromolecule, is very attractive with possible applications in food science, cosmetics, and health care. Lignin

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is a phenyl-propanoid linear oligomeric polyphenol of multifunctional nature.[27,28] More specifically, it contains polar hydrophilic aliphatic and phenolic OH groups within a lipophilic backbone of aryl-propane units.[27,28] Lignin is a material that presents a wide diversity and variability in terms of functional groups depending on its botanical origin and the industrial isolation process. To develop efficient microcapsules, we focused on kraft lignin. This is a lignin residue from the kraft pulping process. Distinct from native lignin, it contains a higher amount of phenolic and a lower content in aliphatic OH groups, a higher molecular weight, and a distinct solubility in alkaline conditions. These physicochemical characteristics should in principle promote capsule formation. In order to produce lignin microcapsules (LMCs), we used a high-intensity ultrasound technology as a tool to emulsify an oil phase in a lignin aqueous solution and subsequently cross-link, at the water/oil interface, lignin functional groups. The mechanism of cross-linking induced by the ultrasonic treatment was elucidated. The addition of a cross-linking reagent for the lignin macromolecules was further explored so as to improve the stability of the lignin shells. In order to evaluate the possibility to use the lignin micro/nanocapsules as a depot and carrier of hydrophobic molecules, a fluorescent molecular probe was dispersed in the oil phase. Coumarin-6 was selected as a model of a hydrophobic drug and a fluorescent dye. Because of its high fluorescent signal, Coumarin-6 has been widely used as a marker in in vitro and in vivo experiments to visualize the brain's uptake of nanoparticles.[29−31] The morphology of the LMCs was studied, and the optimal release conditions of Coumarin-6 were investigated. To evaluate the potential use of lignin capsules in biomedical applications, the interaction of the particles with Chinese hamster ovary (CHO) cell line was examined using the microscopic analyses and biocompatibility assays.

EXPERIMENTAL METHODS

Materials. Kraft Lignin (alkali lignin, low sulfur, CAS number 8068−05−1), poly(ethylene glycol) diglycidyl ether (PEGDEG), M_n = 500 Da, fluorescein isothiocyanate (FITC), Coumarin-6 (C6), hydrogen peroxide solution 30%, sodium dodecyl sulfate (SDS), 2-chloro-4,40,5,50-tetramethyl-1,3,2-dioxaphospholane, *n*-hexane, deuterated pyridine and chloroform were purchased by Aldrich and used as received. Olive oil was obtained from Acros. Milli-Q filtered water was obtained from a Millipore system (18.2 MΩ cm at 25 °C).

Lignin Microcapsules Preparation. All the experiments were performed on lignin alkali solutions in water, with a concentration of 5% w/v and 15% w/v. The pH of lignin solution in Milli-Q water was 9.2. For the ultrasound irradiation, a 750-W High Intensity Microprocessor Controlled Ultrasound (SONIC VCX 750) equipped with a 3 mm-diameter microtip was used at an acoustic power of 160 Wcm^{-2}. After the ultrasound irradiation, the supernatant containing the microcapsules was separated from the remaining solution, mixed with Milli-Q water, and centrifuged at 10 000 rpm for 30 min. The washing was repeated twice.

Ultrasound Production of LMCs. 100 μL of olive oil were added to 1 mL of a lignin alkali solution (5% or 15% w/v). A 3 mm-diameter microtip of a high-intensity ultrasonic horn was positioned at the oil−water interface and the suspension was sonicated for 40s. The emulsion was centrifuged at 10 000 rpm for 30 min, and the supernatant was washed twice with Milli-Q water.

Ultrasound Production of LMC in the Presence of H_2O_2 (H_2O_2−LMC). To 1 mL of the lignin alkali solution, 100 μL of olive oil and 100 μL of H_2O_2 (30% v/v) were added, and the system was sonicated for 40 s. Then, the emulsion was centrifuged at 10 000 rpm for 30 min, and the supernatant was washed twice with Milli-Q water.

Lignin Hydrogel Preparation. To 1 mL of a 35% w/v solution of lignin in water, 95 μL of PEGDEG (lignin/PEGDEG = 2.5:1 w/w) was added. The mixture was stirred for 5 min and left for 8 h at room temperature to obtain a hydrogel. The hydrogel was repeatedly washed with Milli-Q water.

Ultrasound Production of LMC in the Presence of PEGDEG (PEG−LMC). 17.5 and 52.5 μL of PEGDEG were added to 1 mL of lignin alkali solution 5% and 15% w/v (2.5:1 mg of lignin/mg of PEGDEG), respectively. The systems were allowed to react for 12h, at room temperature. Next, 100 μL of olive oil was added, and the suspensions were emulsified for 40 s with the ultrasonic horn. After 15h, the emulsion was centrifuged at 10 000 rpm for 30 min and the supernatant was washed twice with Milli-Q water.

Ultrasonic Encapsulation of Coumarin-6 into LMC. Coumarin-6 (10 mg/mL) was first dissolved in chloroform. Fifty microliters of Coumarin-6 solution was mixed with 50 μL of olive oil. The solution was added to 1 mL of 5% w/v lignin alkali solution and the suspension was emulsified for 40 s with the ultrasonic horn. Then, the emulsion was centrifuged at 10 000 rpm for 30 min and the supernatant was washed twice with Milli-Q water.

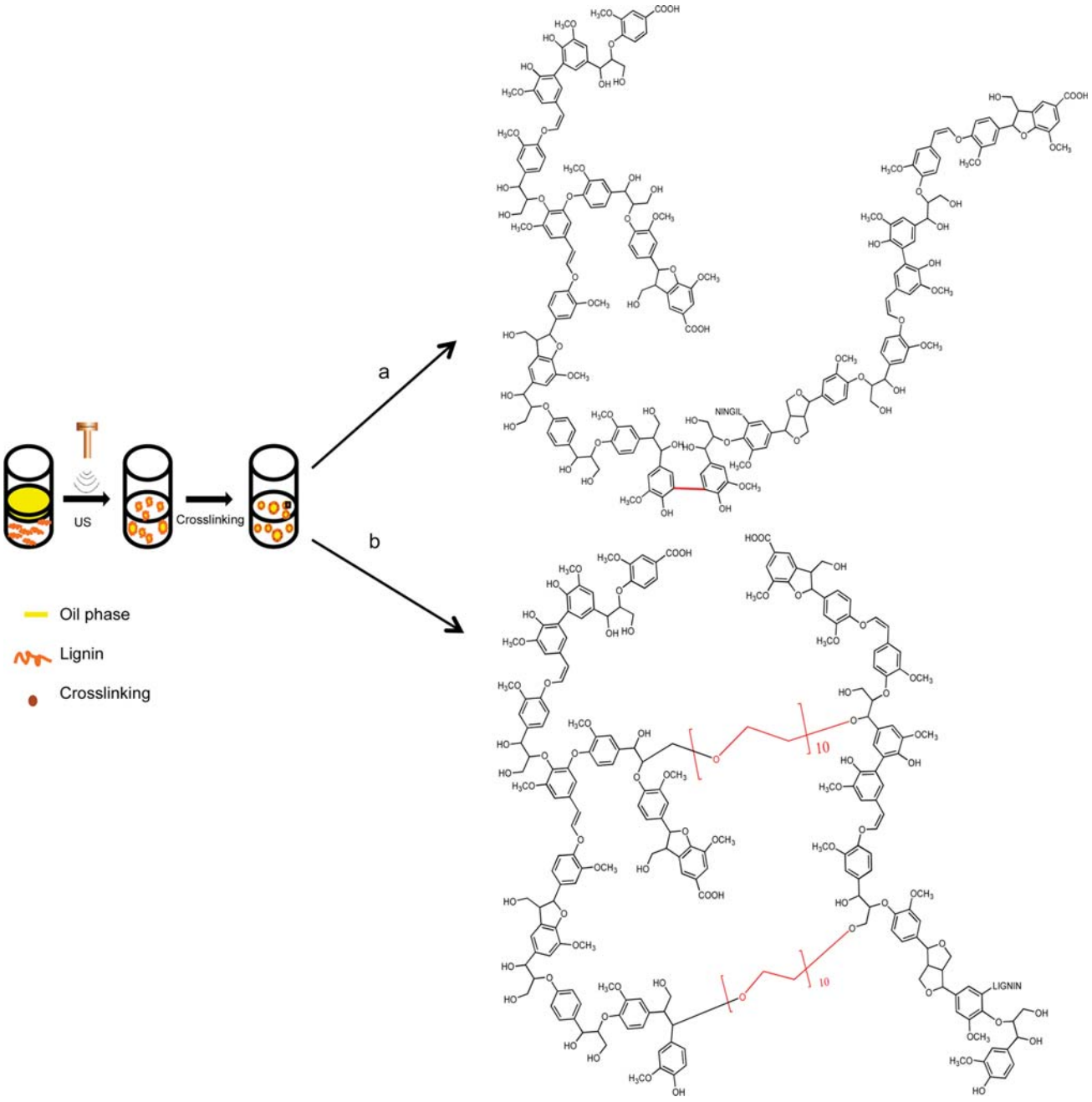
Ultrasonic Encapsulation of Coumarin-6 into LMC−PEGDEG. 17.5 μL of PEGDEG were added to 1 mL of 5% w/v lignin alkali solution and the mixture was allowed to react for 12h. Next, 100 μL of a solution of Coumarin-6 (50 μL of 10 mg/mL solution in chloroform and 50 μL of olive oil) were added to the lignin solution. The suspension was emulsified for 40 s with the ultrasonic horn. After 15 h, the emulsion was centrifuged at 10 000 rpm for 30 min and the supernatant was washed twice with Milli-Q water.

Lignin Characterization. In order to evaluate the structural modification induced in lignin upon the different ultrasonic treatments, the soluble fraction of lignin was separated from the capsules and analyzed. The treated lignin was freeze-dried and analyzed by ^{31}P NMR to characterize the structural modifications induced in phenolic, aliphatic OH, and carboxylic acids.[32−34] The evaluation of the cross-linking efficiency was performed by means of gel permeation chromatography (GPC) analysis. Capsules prepared in the presence of PEGDEG were not analyzed by ^{31}P NMR, as the presence of PEGDEG interfered with the identification of lignin OH groups.

^{31}P NMR Experiments. Derivatization of the sample with 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane was performed as follows. Samples of lignin (30 mg), accurately weighed, were dissolved in a solvent mixture composed of and deuterated pyridine and chloroform, 1.6:1.0 (v/v) ratio (0.4 mL). 2-Chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane (0.1 mL) was then added, followed by the internal standard and the relaxation reagent solution (0.1 mL). The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.[32−34] To improve resolution, a total of 256 scans was acquired. The quantitative ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, while the maximum standard error was $1x\ 10^{-2}$ mmol/g.[32−34]

GPC Analysis. Acetobromination of lignin samples for GPC analysis was carried out as follows.[35] Approximately, 10 mg of lignin was suspended in acetic acid glacial/acetyl bromide mixture (2.5 mL of 92:8 v/v) and stirred at room temperature. After 2 h, the solvent was evaporated under reduced pressure, and then the residue was dissolved in 5 mL tetrahydrofuran (THF). The GPC analyses were performed using a Shimadzu LC 20AT liquid chromatography with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 μL) was injected into a system of columns connected in series (Varian PL gel MIXED-D 5 lm, 1−40 K and PL gel MIXED-D 5 lm, MW 500−20 K), and the analysis was carried out using THF as eluent at a flow rate of 0.50 mL min^{-1}. The GPC system was calibrated against polystyrene standards (molecular weight range of 890−1.86×10^6 g mol^{-1}) as well as lignin monomers and model dimers. In particular, apocynol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxoacetaldehyde were synthesized according to literature procedures and used as monomer and dimer lignin standard, respectively.[36]

Scheme 1. Schematic Representation of LMC Preparation Procedure



Lignin Microcapsules Characterization. Morphological examination of the microcapsules suspension was carried out using a Ziess optical and fluorescence microscope with a 63x and 100X objective lens. The synthesized microcapsules were observed by scanning electron microscopy (SEM) using a field emission apparatus JEOL. Sample of microcapsules was washed with *n*-hexane, centrifuged and then washed with water. After drying, the sample was coated with 200 Å thickness gold−palladium prior to microscopy. Microcapsules average diameter and size distribution were determined with a Horiba LB-550 Dynamic Light Scattering Particle Size Distribution Analyzer, and the experiments were conducted in triplicate.

In Vitro Release Studies of C6-Loaded LMCs. Fifty microliters of Coumarin-6 loaded LMCs was added to 200 μL of a SDS solution 5% w/v. After 30 min of incubation, the suspension was centrifuged at 10000 rpm for 30 min. Then the centrifuged LMCs were removed, and the supernatant was diluted 6 times and filtered over a 0.2 μm filter. The centrifuged LMCs were suspended with 200 μL of fresh SDS solution. The procedure was repeated until no peaks attributable to Coumarin-6 were noticed in the UV spectra of the supernatant. The amount of released Coumarin-6 was evaluated with UV spectroscopy at 472 nm by comparing the absorbance with the calibration curve.

FITC Labeling of LMCs. Six-hundred microliters of the aqueous suspension of LMC was allowed to react with 10 μL of FITC (10 mg/mL) solution in water for 5h at room temperature. The excess FITC was separated by three washes with Milli-Q water.

Internalization of LMCs into Mammalian Cells and Evaluation of Potential Cytotoxicity. In order to evaluate the potential use of LMC in biomedical applications, in vitro internalization of LMC into mammalian cells, cytotoxicity and interference with cell cycle progression were investigated in a CHO cell line. Visualization of FITC-labeled lignin microcapsules into cytoplasm was achieved by immune fluorescent methods and confocal laser scanning microscopy.

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Potential cytotoxicity and interference with cell cycle progression was assessed by analyses of mitotic indices (number of metaphases out of 1000 interphase cells scored expressed as percentage) and the estimation of the frequencies of cells which underwent one (M1), two (M2), or more (M3) replication rounds in the solvent and LMC-treated cells. To discriminate between M1, M2, and M3 cells, DNA was labeled with 5-bromo-2′-deoxyuridine (BrdU), and differential staining of chromatids was obtained using the fluorochrome plus Giemsa staining method. CHO cells ($2n = 22$) were obtained from Dr. J. Williamson (British American Tobacco, UK). For this cell line, karyotype, generation time, plating efficiency, and absence of mycoplasma are checked at regular intervals. Stocks of the CHO cells are stored in liquid nitrogen, and subcultures are prepared from the frozen stocks for experimental use. For this experiment, cells were grown in Eagle minimal essential medium (EMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (1% w/v penicillin and 86 μM streptomycin) and incubated at 37 °C in a 5% carbon dioxide atmosphere and 100% nominal humidity. Approximately 24 h before treatment, an appropriate number of 6-well cell culture plastic multiwell plates was seeded with 2×10^4 cells containing 2 mL complete culture medium and allowed to grow attached on sterile glass cover sleeps. Two parallel cultures were established for each dose-level. The experiment included negative control treated with buffer used to suspend lignin microcapsules (LMC) only. The row A of the multiwell plates was used to show the incorporation of LMC into cytoplasm of cells and row B to assess mitotic indices and the frequencies of M1, M2 and M3 metaphases in the solvent and LMC-treated cultures. For each LMC, a single dose-level (2×10^8 capsules/mL) was employed, and treatment was performed under standard cell culture conditions for 24 h. At the end of treatment, the culture medium was aspirated, and cultures were washed twice before being processed appropriately for confocal laser scanning microscopy or cytotoxicity. Evaluation of mitotic indices and cell cycle progression was performed using conventional cytogenetic techniques and use of optical microscope (Olympus BHA, Japan) at 1024 magnification.

Confocal Scanning Laser Microscopy. Cells treated on coverslips as described above were rinsed in PBS, fixed for 15 min with 3.7% formaldehyde in PBS and dehydrated in ethanol series (70, 90 and 100%) for 3 min. DNA was counterstained with 0.1 μg/mL 4′-6-diamidino-2-phenylindole (DAPI) and coverslips were sealed in antifade solution (Vector). All preparations were examined using a Zeiss laser scanning 710 confocal microscope with Plan-neofluoar ×40/1.30 objective. Two laser excitations lines were used (i.e., 405 nm for DAPI and 488 nm for FITC). Images were collected using Zeiss ZEN software and processed using ImageJ software (NIH-U.S.A.).

RESULTS AND DISCUSSION

Preparation of Lignin Micro/Nanocapsules. Kraft lignin is an amphiphilic biomacromolecule due to the presence of hydroxyl groups and its aromatic backbone. There have been numerous literature citations documenting hydrophobic interactions within lignin.[37] Evidence that lignin components tend to associate has been obtained by evaluating the behavior of kraft lignin under alkaline conditions.[38−42] Additional efforts to evaluate inter and intra molecular association in lignin samples, which are less chemically altered than kraft lignins, include those of Connors et al.,[43] Sarkanen et al.,[44] and Cathala et al.[36] who used Braun's native lignin, organosolv lignins, and milled wood lignin (MWL) and synthetic lignin (DHP), respectively. Alternatively, Cathala et al.[45] examined the association behavior of MWL and lignin model compounds in organic media and concluded that the association of the starting material was not the result of an equilibrium between associated and molecularly dispersed species.

The recent sixe exclusion[37] and light scattering[46] work of Argyropoulos and his team has examined the role of aromatic π stacking within lignin and have further capitalized on this knowledge so as to create a novel analytical technique.[47]

Based on the above knowledge, we embarked on the following strategy to create uniformly spherical kraft lignin microcapsules. We first created an oil emulsion in lignin aqueous solution followed by a high intensity ultrasound assisted cross-linking of lignin at the water/oil interface. As such, we aimed to promote these documented lignin hydrophobic interactions within the oil phase, followed by locking the resulting spherical configurations into place by covalent cross-linking using a high intensity ultrasound treatment.

The abundance of phenolic OH groups in kraft lignin that are able to readily form phenoxy radicals and further cross-link via upon an ultrasound and chemical treatment makes our approach highly appealing since this natural polymer could offer possibilities for the production of such micro/nano containers with tangible commercial ramifications. In this study, we used olive oil as a biocompatible, hydrophobic core to prepare LMCs via an ultrasonic synthetic approach in the presence or absence of cross-linking agents. The production of microcapsules was achieved by sonication of a 5% w/v and 15% w/v of kraft lignin solutions. A schematic representation of the LMC preparation is shown in Scheme 1, route a.

The mechanism responsible for the formation of LMC is a combination of two ultrasound-induced phenomena: emulsification and cross-linking. The first process depends on the collapse intensity of the cavitation bubbles (responsible for the shear forces). The oil-in-water emulsion was obtained applying an acoustic power of 160 Wcm^{-2} for 40 s. The amphiphilic lignin chains are thought to diffuse toward the oil microdroplets so as to stabilize the water/oil interface. In the second step the cross-linking between lignin chains is induced by the hydroxyl (•OH) and superoxide ($HO_2^{-\bullet}$) radicals generated during the acoustic cavitation process.[48] Transient cavitation generates hydroxyl radicals which react with the phenolic groups of lignin and induce oxidation in the lignin backbone.[49] Aiming at the identification of efficient lignin capsules synthesis procedures, different experimental conditions were explored as reported in Table 1. In order to increase the amount of radical species, H_2O_2, a strong oxidizing agent, was added during the ultrasound treatment (Table 1, entries 3,4). Under alkaline conditions, hydrogen peroxide generates •OH radicals, and

Table 1. Codes, Experimental Conditions Used, and Average Size of LMCs

entry	Sample	lignin concentration (w/v)[a]	amount of cross-linking agent (μL/mL)[b]	average size (μm)
1	LMC_5	5%	-	1.1 ± 0.8
2	LMC_15	15%	-	0.7 ± 0.3
3	H_2O_2-LMC_5	5%	100	1.4 ± 1
4	H_2O_2-LMC_15	15%	100	0.8 ± 0.4
5	PEG-LMC_5	5%	17.5	1.0 ± 0.8
6	PEG-LMC_15	15%	52.5	0.3 ± 0.1

[a]After the addition of cross-linking reactants and 100 μL of oil, the suspensions were ultrasound irradiated for 40s, at an acoustic power of 160 W cm^{-2}. [b]The total amount of cross-linking agent added to 1 mL of lignin solution.

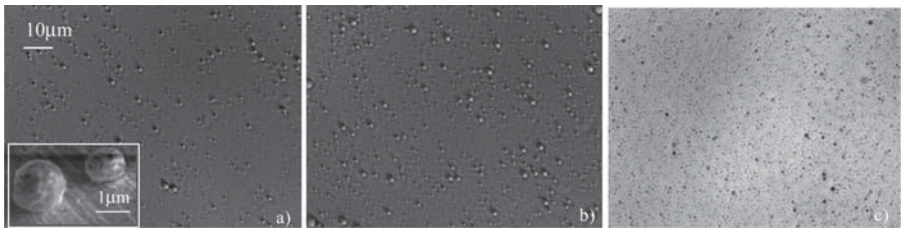

Figure 1. Optical microscopic images of LMC samples: LMC_5 (a), LMC_15 (b), and PEG-LMC_L15 (c). Inset: SEM micrograph of LMC_5.

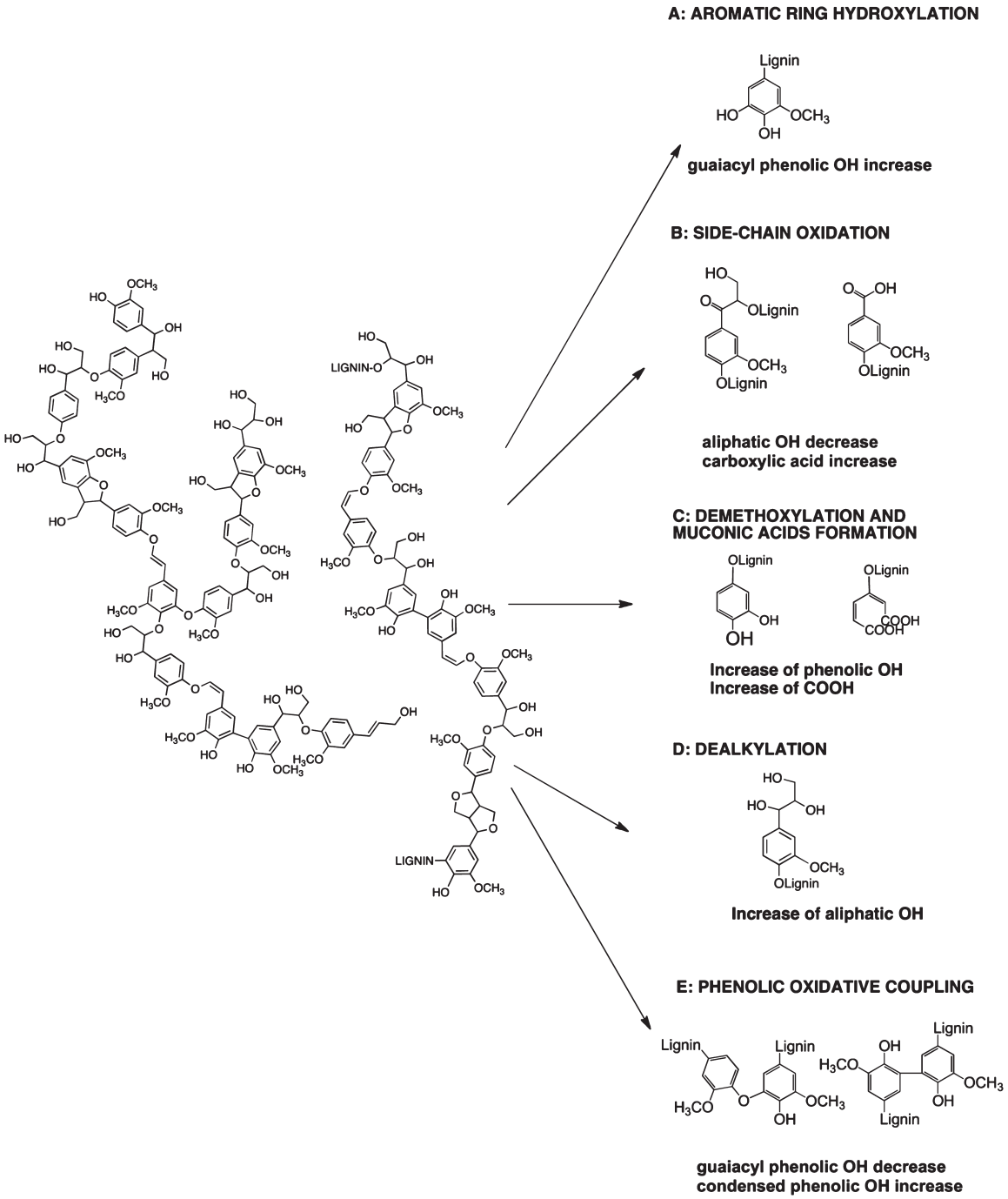

Figure 2. Kraft lignin oxidation pathways in the presence of hydroxy and superoxy radical species at pH 8.

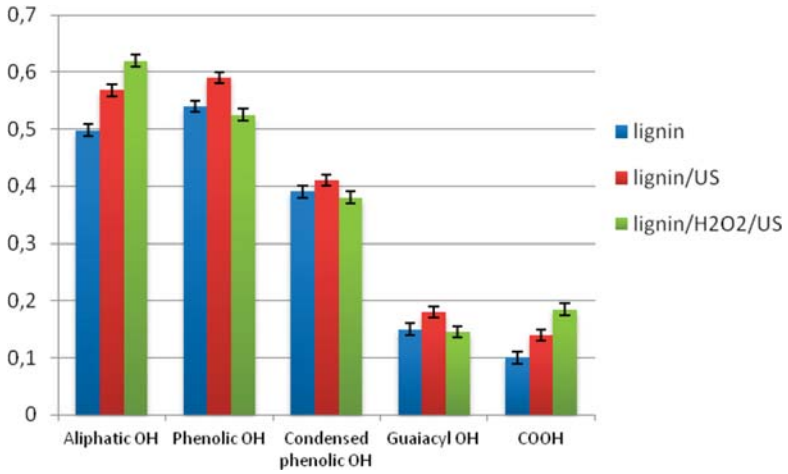

Figure 3. Aliphatic, phenolic OH, and COOH groups occurrence (mmol/g) of lignins treated during capsules preparation. Evaluated by ^{31}P NMR of LMC_5 and H_2O_2-LMC_5 residual lignins.

these additional oxidant species introduced during the microcapsule formation are able, in principle, to promote oxidative coupling reactions within the lignin backbone.[49]

Alternatively, poly(ethylene glycol) diglycidyl ether (PEGDEG)[50] was added as an additional cross-linking agent, before ultrasound treatment of the lignin solution (Table 1, entries 5,6). In this case, to produce the lignin capsules, lignin was preincubated with PEGDEG at the 2.5:1 w/w ratio and below the critical gelation concentration (35% w/w). This allowed slowing down of the gelation kinetic. After 12 h of reaction, oil was added to the mixture, and the emulsion was generated by sonication. Our work showed that when PEG functionalized lignin accumulates at the oil−water interface, effective cross-linking occurs. The possible networking reactions promoted by the ultrasonic treatment of PEG functionalized lignin are shown in Scheme 1, route b. It must be emphasized that in the absence of PEGDEG, the oil phase was not totally enveloped within the capsules since a residual layer of oil was always present after the encapsulation. The layer was removed before centrifugation and purification of the LMCs. However, during the synthesis of PEG-LMC capsules, no evidence of residual oil layer was observed. This suggests that PEG-lignin is more effective in stabilizing the oil phase preventing the oil droplets growth by Ostwald ripening. Both the surfactant and cross-linking properties of poly(ethylene glycol)[50,51] may play a role in improving the encapsulating ability of lignin. It is also of extreme significance to add here that the LMCs produced were stable for over one year in the form of aqueous suspensions. Furthermore, the average size of the capsules did not change during this period.

The morphology of the LMCs was examined by optical microscopy, and it was verified that the produced particles were of a spherical morphology, as one would anticipate based on our conceptual approach described previously. In Table 1, the average size of the particles measured by dynamic light scattering is reported. In Figure 1 the optical images of LMC_5, LMC_15 and PEG-LMC_15 are reported as examples. LMCs have a spherical shape, and their concentration was significantly different for the various synthetic routs. In particular, at a fixed lignin content, the concentration of microcapsules produced using PEGDEG is 10-fold higher than those obtained without using the cross-linking reagent.

The size of the LMCs is approximately 1 μm, except for LMCs prepared in the presence of PEGDEG, whose diameter is 300 nm. To further investigate the surface morphology of LMC microcapsules, SEM microscopy was used. In the inset of Figure 1, the SEM micrograph of LMC_5 is reported as an example. SEM observation confirmed the spherical nature of the microcapsules.

Characterization of the Structural Modification of Lignin Induced by the Ultrasonic Treatment. Ultrasound treatment of aqueous lignin solution in the presence of air results in the formation of hydroxyl and phenoxy radical species that are in a pH-dependent equilibrium and, according to the pH condition, undergo a complex array of different reactions. More specifically, under the mild alkaline conditions of our experimental protocol, the following reactions take place. Hydroxyl radical species undergo aromatic ring hydroxylation, as shown in Figure 2A, and side-chain oxidation processes (Figure 2B). The last reaction pathway implies the introduction of carboxylic moieties in lignin. Superoxy radicals undergo oxidative dealkoxylation reaction (Figure 2C,D). Such reactions can cause demethoxylation and further oxidation of the aromatic rings with formation of muconic acid moieties. Alternatively, they can cause oxidative cleavage of the lignin side chains (Figure 2D). In this case, an overall increase of the lignin aliphatic OH groups will result. Superoxy radical species also generate phenoxy radicals at the phenolic end units. In turn, phenoxy radicals undergo oxidative coupling with an overall increase in condensed OH groups in lignins. (Figure 2E).[22]

The dealkoxylation and aromatic ring cleavage reactions are clearly depolymerizing processes that, besides introducing carboxylic moieties on lignin, cause its fragmentation. Conversely, the oxidative coupling processes allow further lignin polymerization. In order to evaluate the oxidative pathways occurring in our systems, we used quantitative ^{31}P NMR. Such analysis methodology is a powerful technique that allows the identification and quantitative evaluation of the different phenolic, aliphatic, and carboxylic OH groups in a lignin sample after a simple in situ process of phosphitylation with a suitable reagent. Through quantitative ^{31}P NMR acquisition, it is possible to identify the amount of guaiacyl, syringyl, p-hydroxyphenyl, and aliphatic OH groups belonging to residual carbohydrates and to lignin itself, and to evaluate the

amount of condensed lignin subunits such as diphenylmethane and 5−5′ biphenyl groups. However, it was not possible to perform ^{31}P NMR spectroscopy of LMC suspensions due to the presence of residual oleic acid that overlaps with the lignin signals and does not allow quantitative integration of the phenolic OH signals. For this reason, in order to clarify the general reaction pathways involved in the lignin capsules, the structure of lignin remaining in the solution after LMCs preparation, about 85% w/w of the starting lignin, was studied. ^{31}P NMR of freeze-dried LMC_5 and H_2O_2-LMC_5 lignin samples dissolved in deuterated pyridine, and chloroform was used to determine the various structural modifications. In Figure 3, the occurrence of aliphatic −OH groups, condensed phenolic OH, and carboxylic moieties in the starting lignin, LMC_5 and H_2O_2-LMC_5 samples are reported. The aliphatic OH groups concentration increased upon treatment with US. In the presence of hydrogen peroxide, such increase was further improved. This indicates the occurrence of dealkylation reactions (reaction pathway 2D). The condensed phenolic OH group concentration slightly increased after the US treatment. This is indicative of oxidative coupling and cross-linking as reported in Figure 2E. The increase of carboxylic acid concentration in the treated samples shows the occurrence of side-chain oxidation processes and aromatic ring cleavage (Figure 2B,C).

Thus, one may conclude that three different processes are simultaneously occurring: (i) introduction of carboxylic moieties, (ii) lignin fragmentation, and (iii) lignin cross-linking. The NMR spectrum of a H_2O_2-LMCs lignin sample showed a similar behavior with a more pronounced effect when compared to LMCs, due to the stronger oxidative conditions exerted by the hydrogen peroxide (Figure 3). Hence, the stabilizing properties of the lignin shell of LMC_5 could be ascribed to both the increased amphiphilicity of the lignin chains and chemical cross-linking via phenolic condensation. The proposed hypothetical structure is reported in Scheme 1a.

These data highlight that the ultrasound treatment of lignin promotes its self-organization and dense packing of the macromolecules around the organic phase.

In order to evaluate the extent of the different oxidative processes, we monitored the distribution of the LMCs molecular weights by GPC. Table 2 shows the M_n and M_w values of the starting lignin, ultrasound-treated 5% lignin, and 5% PEG-functionalized lignin.

Table 2. Molecular Weight Distribution of Lignin and LMCs

sample	M_n	M_w
KL	9400	47 000
LMC5	21 000	157 000
PEG LMC5	31 500	174 000

Compared to the untreated lignin, both the M_n and M_w increase for LMCs, and the PEG−LMCs show the significant occurrence of coupling reactions. The highest M_n value was found when PEG was added to the system to form the LMCs, confirming the ability of PEGDEG to cross-link the lignin chains.

Drug Loading and Drug Release Study. LMC-5 and PEG-LMC_5 were employed for the loading of the hydrophobic fluorescent probe Coumarin-6. The loading of the hydrophobic molecule was obtained by adding Coumarin-6 dissolved in chloroform to the oil phase during LMCs formation. In Figure 4, the optical fluorescence images of the two loaded samples are shown.

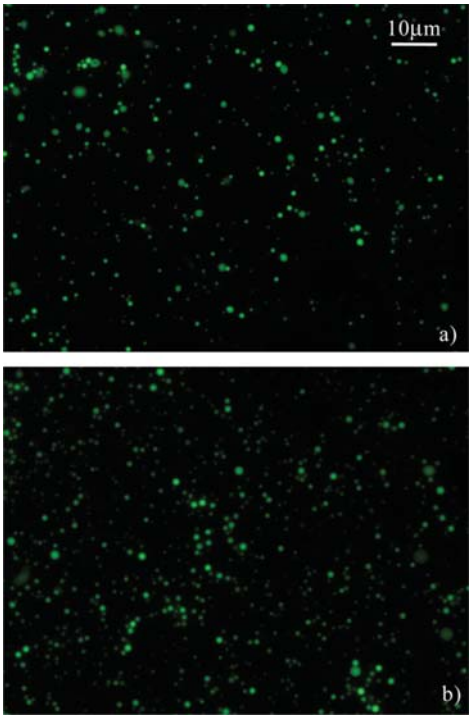

Figure 4. Fluorescence microscopy images of Coumarin-6-loaded LMC_5 (a) and PEGLMC_5 (b).

No significant release of Coumarin-6 from the LMCs suspension in water was observed up to a month. Aiming to use LMCs for cosmetic and topical applications, the release of the hydrophobic molecule in the presence of a surfactant was investigated. The Coumarin-6 release in the presence of SDS solution 5% w/v was studied by means of UV-spectroscopy. In Figure 5, the kinetic release curves of both investigated samples are reported.

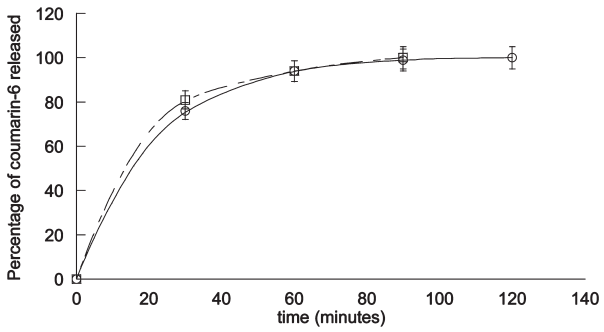

Figure 5. The Coumarin-6 release profile of LMC_5 (○) and PEG-LMC_5(□), by addition of a 5% solution of SDS.

Under this condition, both LMC-5 and PEG-LMC_5 showed that almost 100% of entrapped Coumarin-6 was released in 60 min. Both samples released their cargo with a similar rate. The amount of released Coumarin-6 from PEG-LMC_5 was 1.5 times higher than that released from LMC-5. It must be noted that not all the oil phase is generally embodied in LMC-5, while in the PEG-LMC_5 sample, almost all the oil

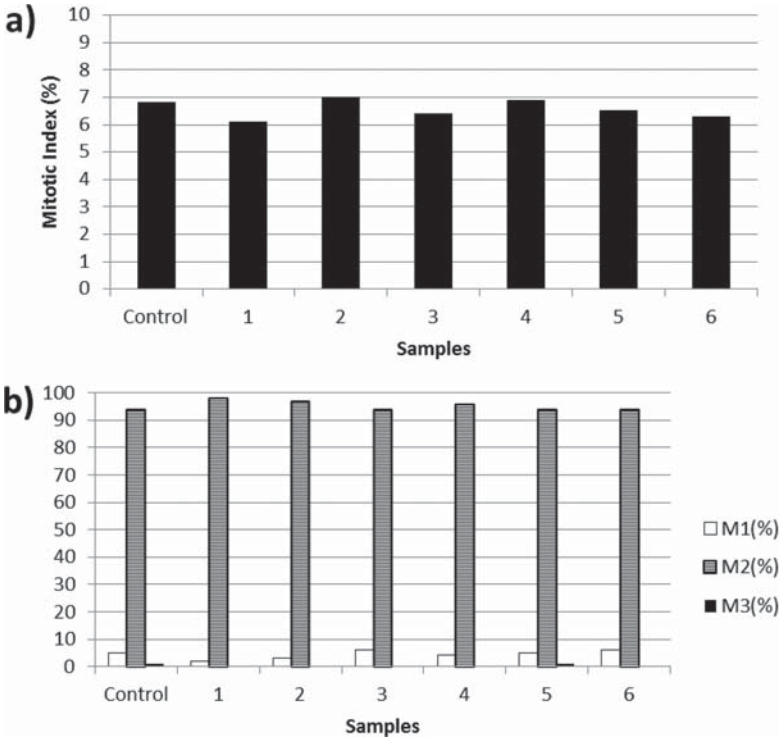

Figure 6. (a) Mitotic index values in solvent control and LMC treated cultures. (b) Frequencies of cells which underwent one (M1), two (M2), or more (M3) replication rounds in solvent control and LMC treated cultures. 1 = LMC_5; 2= LMC_15; 3 = H_2O_2-LMC_5; 4 = H_2O_2-LMC_15; 5 = PEG-LMC_5; 6 = PEG-LMC_15.

phase is embedded by the lignin shell. As mentioned, the role of PEG is presumably to better promote the location of the lignin-PEG chains at the oil−water interface, which in turn allows one to obtain a higher amount of microcapsules and endows PEG-LMC_5 microcapsules with higher encapsulation efficiency.

The release of Coumarin-6 from the lignin capsules, in the presence of SDS, can be attributed to two concomitant causes: a weakening of the lignin hydrophobic interactions, and a higher affinity of the molecule for the new dispersing fluid. The SDS molecules interfere with the stabilizing properties of lignin at the core−shell boundaries and trigger the release of Coumarin-6 because of the higher solubility of the hydrophobic molecule in SDS Solution. These results highlight the possibility to use lignin micro/nanocapsules for topical application as reservoir for skin defender ingredients (anti-oxidants and essential oils could be examples), released when in contact with the lipid tissue or dermatological diseases. Moreover, we can further invoke the use of LMCs as a carrier for the delivery of hydrophobic molecules versus surfactants present in the alveoli of lungs or to the mucosal barrier of gastric surfactant present in the human stomach, in order to treat lung and ulcer diseases.

Biocompatibility of LMCs. To assert the potential use of LMCs in cosmetic and/or biomedical fields, in vitro biocompatibility studies of lignin capsules were performed. No cytotoxicity or interference with cell cycle progression by treatments with LMC were observed since mitotic index values and frequencies of M1, M2, and M3 cells were very similar to those observed in solvent treated cultures (Figure 6a,b).

To determine whether LMCs were efficiently internalized by CHO cells, their intracellular distribution was investigated by confocal laser scanning microscopy (CLSM) analyses. Results obtained indicated that all different LMC were efficiently internalized into the cytoplasm of CHO cells. An example of "merged" microphotographs is shown in Figure 7. LMC,

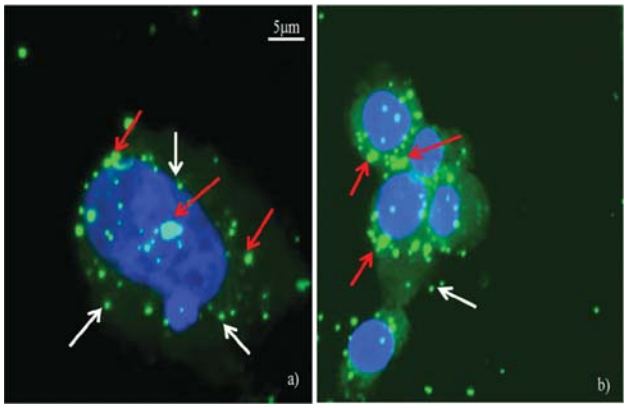

Figure 7. Internalization of LMC_5 (a) and LMC_15 (b) into cytoplasm of CHO cells. Nuclei are stained in blue (DAPI), and cytoplasm is the light diffused greenish fluorescent area. White arrows indicate well-preserved spherical LCMs, and red arrows indicate disassembled lignin shells.

generally preserved their spherical configuration following cellular uptake (Figure 7 B, arrows) and rarely showed an altered spotty fluorescent shape, suggestive of disassembling of the lignin shell. Although the mechanism of internalization was not investigated in this case, it is widely recognized that the micrometer-sized particles gain entry into cells via endocytosis (macropinocytosis),[52] and their spreading in the cytoplasm

(Figure 7a,b) indicates that LMCs are able to escape the endocytic vesicles and access the cytosol.

CONCLUSIONS

Oil-filled lignin microcapsules were successfully prepared by the use of ultrasound. Further, H_2O_2 or telechelic poly(ethylene glycol) were used to induce stronger oxidative conditions or as a cross-linking agent, respectively. According to quantitative ^{31}P NMR analyses, we can assert that the ultrasound irradiation involves three different processes: the introduction of carboxylic moieties, lignin fragmentation, and lignin cross-linking. The presence of carboxylic moieties and an increase of the lignin aliphatic OH groups are responsible for an increased amphiphilicity of lignin, and consequently allow the packing of lignin macromolecules around the organic phase. Furthermore, oxidative coupling of phenoxy radicals induce lignin polymerization. In the PEG-LMCs, the flexible chains of the reactive PEGDEG allow the lignin molecules to cross-link and increase their molecular weight. Morphology of micro/nanocapsules showed spherical shape with an average size diameter in the range of 0.3−1.1 μm.

Coumarin-6 was loaded during LMC preparation as a model hydrophobic drug, and its release in SDS was studied. The drug release was exclusively affected by the release medium conditions and not by the structure of LMC. Biocompatibility studies of LMCs with CHO cells provided promising evidence for their potential use in the biomedical field, thus creating novel avenues for the utilization and of a valuable biobased resource material.

ASSOCIATED CONTENT

Supporting Information

^{31}P-NMR spectrum of kraft lignin and GPC profiles of kraft lignin and lignin capsules, KL, LMC_5, and PEG LMC_5, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: crestini@stc.uniroma2.it.

Author Contributions

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Notes

The authors declare no competing financial interest.

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