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Novel biodegradable laminarin microparticles for biomedical applications

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Abstract

Fabrication of biocompatible polymeric carriers for a sustained/controlled drug-delivery have been extensively explored over the years. Furthermore, systems based on polymers from natural origins exceed conventional polymers in biocompatibility, biodegradability and cost efficiency. Polysaccharides are one of the most common biopolymers found in nature and they can achieve a high degree of complexity and fine biological properties. Herein, is proposed a biodegradable and biocompatible microcarrier synthesized from laminarin, a low Mw marine polysaccharide based on glucose units with a great biological activity, such as, immune modulation and antimicrobial properties. Within this work, controlled size microparticles were obtained from novel modifications of laminarin. Microparticles shown 40% release of fluorescein isothiocyanate-dextran (70 kDa) after 24 h and full degradability after 11 days, when in physiological conditions. When incubated with human adipose stem and L929 cell lines (up to a microparticle concentration of 100 µg/mL) no cytotoxicity was perceived, and neither membrane or nucleus disturbance. Thus, microparticles synthesized from laminarin, proved to be a cost efficient, biocompatible and biodegradable system.

Keywords: Marine polysaccharides, laminarin, microparticles, biocompatible, biodegradable, L929 & hASCs.

1. Introduction

Polysaccharides particles are excellent biocompatible and biodegradable carriers with an array of possible bioactivities according to their structure, thus augmenting certain drugs effects [1-6]. Different techniques can be used to produce several size particles and accordingly, the modification of the biopolymer and its charge hold important factors. For instance, modifications with hydrophobic moieties allow a self-assembly into micelles, which may be excellent carriers for poor water-soluble drugs[7, 8]. Different type of processes can be applied after the emulsification process to produce polysaccharides carriers with different sizes, namely, solvent diffusion[9], solvent evaporation[10, 11], photo-reticulation[12, 13] and the use of superhydrophobic surfaces[14, 15]. Biopolymers, such as polysaccharides are used as stabilizers in the development of food emulsions, avoiding the aggregation of micelles. Hence, it is possible to replace surfactants, solid particles or proteins that are commonly used as emulsifying agents[16, 17]. Furthermore, a system based on natural polysaccharide and immiscible polymer blends has proven to be a good approach for the production homogeneous and degradable polysaccharides microparticles using mild conditions[18]. Accordingly, proteins, photosensitive and thermosensitive drugs can be encapsulated in this type of carriers, due to unnecessary use of organic solvents, temperature or irradiation.

Laminarin (LAM), also called laminaran, is a neutral small polysaccharide (Mw <10 kDa; 20 to 50 units of glucose) and is the main energy storage polysaccharide found in brown algae[19], a marine specie rich in different elements important for biomedical applications[20-24]. Mainly due the important biological activity aspects such as, immune modulation[25, 26], antioxidation[27, 28] and antimicrobial properties[27]. Additionally, some authors reported the capability of LAM to trigger apoptosis on different human colon cancer cell lines[29-31]. LAM has a linear structure with a degree of side branch through the β-(1,3; 1,6) bonds between glucose units. Further, the ratio between both bonds may differ between algae specie and form a triple helical conformation[32, 33], which may difficult their modification. There are only few examples in the literature surrounding the modification of LAM, either by sulfonation or by adding a methacrylic or propargyl moieties. LAM sulfonation has shown to significantly enhance the antitumor activity of LAM [34], while methacrylation of LAM has been used to obtain microparticles and hydrogels for biomedical applications[35-38]. Furthermore, the modification with propargyl was used in a Cu(I) catalyzed Huisgen cycloaddition in a way to synthesize nanoparticles for drug delivery through the interaction between the polysaccharides and proteins[24]. Besides particles and hydrogels, LAM has also been used to produce regular size and shape organized layered nanostructures similar to the natural process of biomineralized structures found in nature[33].

In this study is reported the modification of LAM with alkyne and azide moieties as a way of polymerization trough the formation of a triazole linker using click-chemistry. The obtained modified polymers were characterized by infrared and ^{1}H-NMR spectroscopy. The combination of Cu(I) catalyzed Huisgen cycloaddition and micro-emulsification of immiscible polymers, are combined to synthesize novel LAM biodegradable microparticles. Accordingly, when a stable aqueous biphasic system is disturbed, a microemulsion is created. Then, the Cu (I)

catalyzed Huisgen cycloaddition enables the formation of solid microparticles. This type of procedure has been used before for the synthesis of degradable microparticles of dextran[18]. LAM microparticles morphology and size dispersity was assessed by scanning electron microscopy (SEM) and fluorescent microscopy. Microparticles' degradability and release profile of macromolecule chains attached with a fluorophore were performed in physiological conditions and used to prove the degradability and sustain release of such microcarriers. Moreover, microparticles biocompatibility were tested through live-dead assays with two different cell lines (human adipose stem cells and fibroblasts mouse cells). As far as the authors know it is the first time that is reported the modification of LAM with azides, their assembly into biodegradable LAM microparticles and their consequent characterization.

2. Experimental

Materials: Used reagents and materials in this work were purchased from Sigma-Aldrich, Fluka, Merck, Fisher Scientific, Sarstedt and Spectrum Labs at analytical grade and used without any further purification.

Instrumentation: ^{1}H spectra was recorded on a Bruker Avance-300 spectrometer at 300.13 MHz. Tetramethylsilane was used as internal reference. Fourier-transform infrared spectroscopy (FTIR) spectra were recorded using an IR-FT Mattsson 7000 galaxy series with a Golden Gate ATR. Microscopy images were obtained using a Zeiss Axio Imager 2 fluorescence microscope. Confocal laser scanning microscopic (CLSM) images were acquired using a Zeiss laser scanning microscope 880 Airyscan. Morphological images were obtained using Hitachi SU-70 SEM. Particle size distribution analysis was taken by numerical assessment of SEM and microscopy fluorescence images using *ImageJ* software.

Synthesis of 3-azidopropanol (AP). 3-Bromopropanol (10.0 g; 72 mmol) and sodium azide (7.66 g; 118 mmol) were dissolved in a mixture of acetone (120 mL) and water (20 mL) and stirred at 65 °C overnight. Acetone was removed under reduced pressure and 100 mL of water was added to the mixture. The mixture solution was three times extracted with 100 mL diethyl ether and dried over sodium sulfate. The diethyl ether was removed by rotary evaporation and AP was obtained as a colorless oil (5.2 g, η = 71%). **^{1}H NMR** (300.13 MHz, $CDCl_3$): δ (ppm) 1.73 – 1.82 (m, 2H, C-CH2-C), 3.39 (t, 2H, *J* = 6.6 Hz, CH_2-N_3), 3.67 (t, 2H, *J* = 6.0 Hz, CH_2-OH).

Synthesis of 3-azidopropyl carbonylimidazole (AP-CI). A dry round bottomed flask was charged with 18.5 g (114 mmol) of 1,1'-carbonyldiimidazole (CDI) and 200 mL ethyl acetate yielding a turbid suspension. AP (7.1 mL, 76 mmol) was added dropwise under vigorous stirring while the reaction mixture turned into a clear solution. After 2 h reaction at room temperature, the solution was extracted three times with 200 mL of water. The organic layer was dried over sodium sulfate, evaporated by rotary evaporation obtaining AP-CI as a pale-yellow oil (10.1 g, η = 68%) **^{1}H NMR** (300.13 MHz, $CDCl_3$): δ (ppm) 1.92–2.06 (m, 2H, CH_2-CH_2-CH_2), 3.42 (t, 2H, *J* = 6.5 Hz, CH_2-N_3), 4.44 (t, 2H, *J* = 6.2 Hz, CH_2-O), 6.90–7.03 (m, 1H, C=CH-N), 7.35 (t, 1H, *J* = 1.4 Hz, N-CH=C), 8.06 (s, 1H, N-CH=N).

Synthesis of laminarin-azidopropylcarbonate (LAM-N_3). In a dry round bottomed flask 1 g of LAM (corresponding to 5.56 mmol glucose repeating units) was dissolved in 20 mL anhydrous dimethyl sulfoxide (DMSO). To this mixture 214 mg (1.1 mmol) of AP-CI was added and the reaction was stirred overnight at 50 °C under a N_2 atmosphere. The reaction solution was transferred to dialysis bags with a molecular weight cut-off (MWCO) of 3.5 kDa and dialyzed for 5 days under stirring against distilled water, while the dialysis solvent was changed twice a day. Purified LAM-N_3 was obtained by lyophilization as a white fluffy powder with a degree of substitution (DS; i.e. the number of azide moieties per 100 glucose units) equal to 11% and stored at a dry place protected from light. **^{1}H NMR** (300.13 MHz, DMSO): δ (ppm) 1.79-1.95 (m, 2H, CH_2-N_3), 2.90-3.88 (m, LAM backbone), 4.08-4.28 (m, 2H, CH_2-O), 4.30-5.62 (m, LAM backbone).

Synthesis of propargyl carbonylimidazole (PA-CI). A dry round bottomed flask was charged with 14.60 g (90 mmol) of CDI and 100 mL dichloromethane yielding a turbid suspension. 2.90 mL (50 mmol) of propargyl alcohol (PA) was added under vigorous stirring yielding a clear solution upon dissolution of the PA. After 1 h reaction at room temperature the mixture was extracted three times with 100 mL water. The dichloromethane was removed by rotary evaporation and PA-CI was obtained as a dry powder (5.6 g, η = 75%). **^{1}H NMR** (300.13 MHz, $CDCl_3$): δ (ppm) 2.62 (t, 1H, *J* = 2.5 Hz, HC≡C), 4.99 (d, 2H, *J* = 2.5 Hz, CH_2-O), 7.03-7.1 (m, 1H, C=CH-N), 7.44 (t, 1H, *J* = 1.4 Hz, N-CH=C), 8.15 (s, 1H, N-CH=N).

Synthesis of laminarin-propargylcarbonate (LAM-C≡C). In a dry round bottomed flask 1 g of LAM (corresponding to 5.56 mmol of glucose repeating units) was dissolved in 20 ml of anhydrous DMSO. To this mixture 169 mg (1.1 mmol) of PA-CI was added and the reaction was stirred overnight at 50 °C under N_2 atmosphere. The reaction solution was transferred to dialysis bags (MWCO 3.5 kDa) and dialyzed for 5 days under stirring against distilled water, while the dialysis solvent was changed twice a day. Purified LAM-C ≡ C was obtained by lyophilization as a white fluffy powder (DS = 14%) and stored at a dry place protected from light. **^{1}H NMR** (300.13 MHz, DMSO): δ (ppm) 2.28 (m, 1H, HC≡C), 2.92-4.67 (m, LAM backbone), 4.72-4.86 (m, 2H, O-CH_2-C≡), 4.89-5.70 (LAM backbone).

Fabrication of LAM microparticles. Microparticles were fabricated using a microemulsion method based on the immiscibility between an aqueous LAM phase and polyethylene glycol phase[18]. 12.5 mg of LAM-N_3 and 9.8 mg of LAM-C≡C were dissolved in 210 µL of MilliQ water, as a way to obtain 1:1 ratio of the modified LAM moieties. This solution was added to 268 µL of a 50% (w/w in pure water) polyethylene glycol (8 kDa). A solution of $CuSO_4.5H_2O$ (50 mg/mL) in water was added to an ice-cold solution of sodium ascorbate (50 mg/mL) in water. To the reaction mixture, 34 µL from the resulting Cu(I) solution was added, and vortexed for 60 s. The reaction was allowed to proceed for 30 min, with no further stirring or vortex. After that, 2 mL of water was added to quench the reaction and the resultant was centrifuged (5000*g*/10 min) 3 times with washing steps using 2 mL of water. Finally, the obtained LAM microparticles were freeze-dried and stored protected from light. In order to incorporate a macromolecule as an imaging agent, 2.5 mg of fluorescein isothiocyanate-dextran (FITC-dextran, 70 kDa) was added onto the 210 µL of water prior to the microemulsion step. Microparticles were achieved with an average of 71% yield and 85% of FITC-dextran incorporation.

Microparticles mass loss assay. Degradability essays were conducted by creating a suspension of 0.6 mg / mL in phosphate-buffered saline (PBS) at pH 7.4 and placing them in a heating bath (37 ºC at 80 rpm) to mimic physiological conditions. The samples after each time point (1, 3, 7 and 11 days) were freeze-dried for 2 days and weighted. Microparticles NMR solution were performed by dissolving 10 mg of the microparticles into a 500 µL solution of D_2O/NaOD (95/5) at pH 12.

Microparticles degradability. Microparticles degradation was carried out in PBS solution at 37 ºC under

physiological pH (7.4). To understand the influence of culture medium on particles degradation the same assay was performed using Dulbecco's modified Eagle's medium low glucose (DMEM-LG) and alpha modification of Eagle's minimum essential medium (α-MEM), respectively. Briefly, particle suspensions at a concentration of 2 µg/mL were incubated in a µ-slide 8-well plate (ibidi GmbH) for 72 h. Different conditions were tested, i.e., α-MEM medium with and without fetal bovine serum (FBS) and DMEM-LG with and without FBS, in PBS and in MilliQ water with exact concentration of carbonate ions present in DMEM-LG (26 mM) or in α-MEM (44 mM).

Microparticles release studies. Encapsulation of FITC-dextran (70 kDa) into the microparticles was quantified by centrifuging the particles (5000*g*/10 min) after synthesis and reading the absorbance at 490 nm of the supernatant. Release profile was drawn by creating a suspension of 25 mg of the microparticles loaded with 2.5 mg of FITC-dextran in 5 mL of PBS (at pH 7.4). The experiment was conducted for 11 days, in a heating bath at 37 ºC and at a stirring velocity of 80 rpm, till achieve microparticles full degradability. For each time point, the sample was centrifuged (5000*g*/10 min) and then 0.5 mL of the supernatant was taken and replaced with 0.5 mL of fresh PBS. The samples were frozen at -20 ºC and protected from the light. To analyze, the samples were defrosted, and the absorbance at 490 nm was measured on a microplate reader.

SEM. This technique was utilized as a mean to evaluate the size and morphology of the biodegradable LAM microparticles. After freeze-drying, the particles were deposit on a metal stub using an ethanol dispersion that was dried for 1 day at room temperature. The sample was then sputtered in gold and analysed in a Hitachi SU-70. Unmodified LAM morphology was assessed by dissolving LAM in deionised water and by applying 1 µL on a polished silicon surface, letting it dry and repeat, after a couple of cycles the sample was cover with carbon and analysed in a Hitachi SU-70.

Cell Seeding. L929 mouse fibroblasts (ATCC ® CRL-6364™) and human adipose stem cells (ATCC® PCS-500-011™) (hASCs) were seeded in a tissue culture flasks T-175 (Sarstedt) using DMEM-LG and α -MEM (supplemented with 10% FBS and 1% antibiotic/antimycotic) under controlled atmosphere of 5% CO_2 and 37 °C, respectively. Upon reach confluence, cells were trypsinized and seeded in contact with different microparticles concentrations (100, 10, 1 µg/mL) at a density of $1x10^4$ cells mL^{-1} on 96-well plates for 72 h. Besides the cell incubation with the micros, controls were made using the copper concentration utilized in the synthesis, unmodified LAM (100 µg / mL) and a control without particles.

Live-dead staining. Cell viability in contact with the microparticles was evaluated by incubating the different concentrations with Calcein-AM/propidium iodide (PI) (live-dead Kit, ThermoFischer Scientific) for 20 min, according to the manufacturer's protocol. This assay was performed for the 24 and 72 h timepoints. Fluorescence CLSM was used to image the stained cells. Acquired data was processed in Zeiss ZEN v2.3 blue edition software. Live/dead results quantification was performed by *ImageJ* software analysis.

Cell Morphology Analysis. Cell morphology was assessed by imaging cells in contact with the microparticles using fluorescence CLSM. To do so, cells were fixed by incubating them in a 4% solution of paraformaldehyde for 20 min, as previously reported[39]. Then, cell structures, namely, membrane and nucleus were labelled using wheat germ agglutin (WGA) (1:100 in Dulbecco's phosphate-buffered saline (DPBS)) solution for 20 min, and 4´,6-diamidino-2-phenylindole (DAPI) (1:1000 in DPBS) solution during 15 min, respectively. The samples were then analyzed at 24 and 72h timepoints.

3. Results and Discussion

Synthesis of AP, AP-CI and PA-CI. AP was synthesized according literature and has been successfully synthesized by S_N2 type reaction from the azide anion as an excellent nucleophile and the alkyl halide in 77% yield[40] (Scheme 1). The chemical structure of AP was confirmed by ^{1}H NMR spectroscopy where the aliphatic protons appeared at 1.77, 3.37 and 3.69 ppm (Figure S1). 3-azidopropyl carbonylimidazole (AP-CI) and propargyl carbonylimidazole (PA-CI) were obtaining according procedure in literature, by activating PA and AP, respectively with CDI[18] (Scheme 1). The final chemical structures of AP-CI and PA-CI were also confirmed by ^{1}H NMR spectroscopy (Figure S2 and S3). The alkyne proton of PA-CI appeared at 2.62 ppm, while the aliphatic protons of AP-CI appear at 2.00, 3.42, and 4.44 ppm. The aromatic protons of the diazole ring for AP-CI and PA-CI appear in the form of multiple signals around 7.0, 7.4 and 8.1 ppm confirming the expected structure.

Synthesis of LAM-N_3 and LAM-C≡C. LAM-N_3 and LAM-C≡C were synthesized with a DS of 11 and 14, respectively, as determined by ^{1}H-NMR spectroscopy. LAM-N_3 and LAM-C≡C were obtained by reacting LAM with activated carbonylimidazoles (AP-CI and PA-CI) in DMSO at 50 ºC under nitrogen atmosphere conditions (Scheme 1), resulting in the formation of a carbonate ester between the LAM backbone and the carrying azidopropyl (LAM-N_3) and propargyl (LAM-C≡C) moieties, respectively. Also, by ^{1}H NMR spectroscopy is possible to see the changes in the LAM backbone. The conjugation of PA-CI with the LAM backbone was confirmed by the appearance of the alkyne proton signal at 2.28 ppm for LAM-C≡C (Figure 1). Concerning the insertion of the AP-CI for the formation of LAM-N_3, a multiplet signal corresponding to CH_2-N_3 was found at 1.79-1.95 ppm. By comparison (Figure 2A), in the FTIR spectrum of LAM and the respective derivatives, is possible to confirm the vibration of the azide group at 2170 cm^{-1}. At 1270 cm^{-1} (C-O) and 1750 cm^{-1} (C=O) is also possible to confirm the vibrations of the esters groups peaks and regarding the propargyl modification the C≡CH, and C≡C vibration may be masked by the other vibrations, but the esters peaks are still visible at 1270 (C-O) cm^{-1} and 1750 (C=O), respectively.

Fabricating LAM Microparticles. LAM has proven to have attractive properties for biomedical applications[38] and so, carriers based on such polysaccharide may provide new ways of drug-delivery with low side-effects. LAM was modified with azide and alkyne groups in order to obtain microparticles through microemulsion combined with a Huisgen cycloaddition

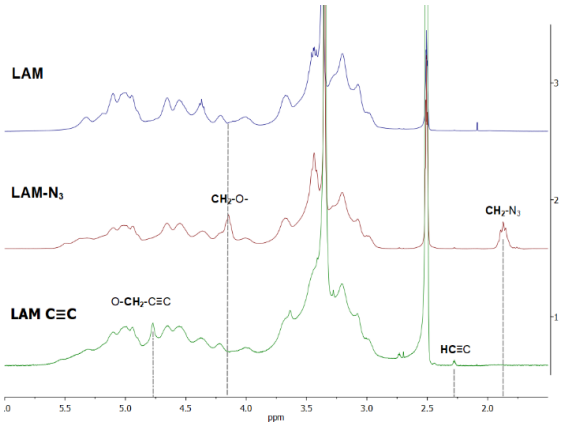
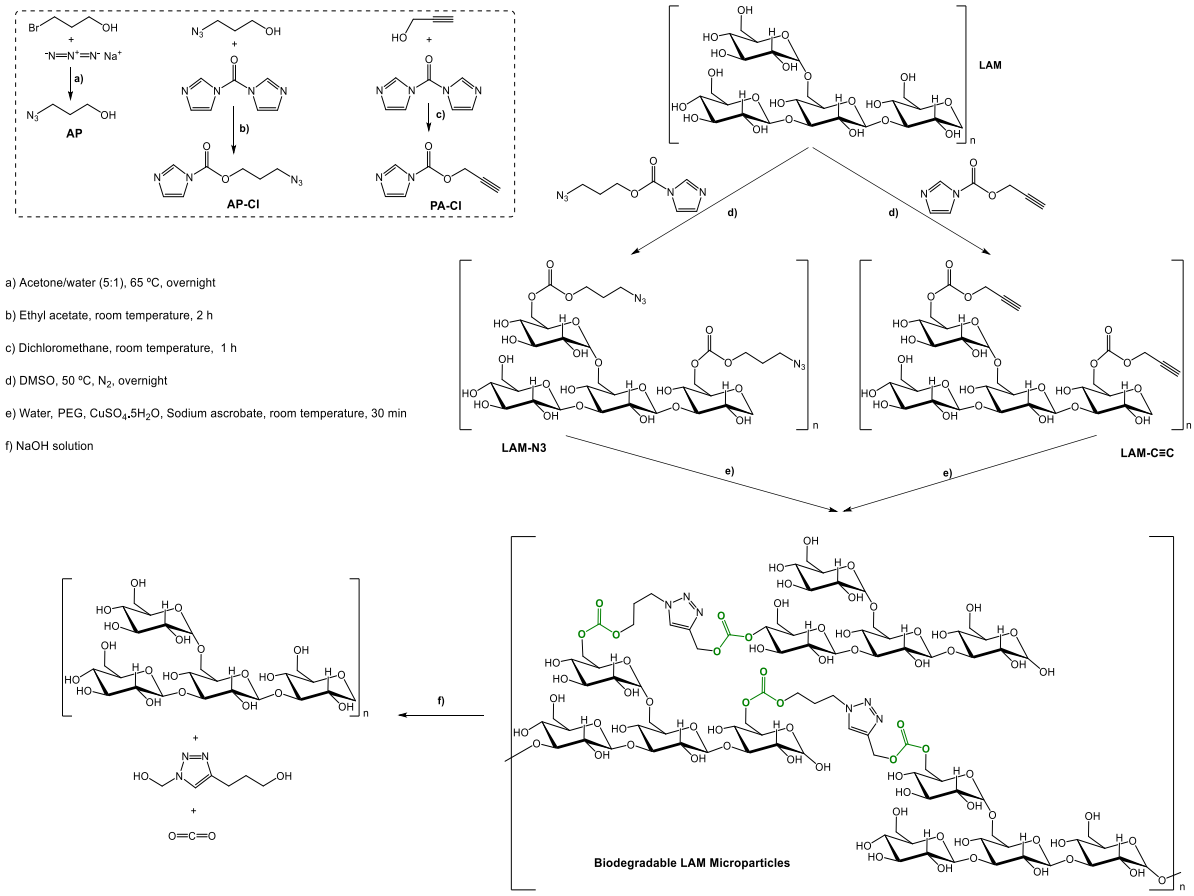


Figure 1 – Characterization of the microparticles precursors by ^{1}H-NMR in DMSO. LAM (unmodified); LAM-N_3 (modified with azide); LAM-C≡C (modified with propargyl).



Scheme 1 - Synthesis of carbonylimidazole (AP, AP-CI, PA-CI) and laminarin (LAM-N_3, LAM-C≡C, LAM microparticles) derivatives and their reaction conditions. Highlighted at green the cleavable bonds at pH above 7.

reaction catalyzed by copper (I). Particle size was evaluated through both fluorescence microscopy and SEM. By fluorescence microscopy analysis was, shown that the particles seemed to emit a low auto-florescence with an average size of 15 ± 4 µm (Figure S4). Similarly, SEM allowed to understand that the particles were spherical, monodispersed, with a smooth surface (Figure 2B and 2C) and an average size of 7 ± 4 µm (Figure S5). The difference in the results may be explained by an auto-of-focus fluorescence effect present in the fluorescence microscope, thus indication a higher size than expected. This procedure had previously been developed and used in higher molecular weight polymers, like dextran[18]. As the synthesis were performed through the usage of esters, the microparticles have an enhanced biodegradability, since this groups are known to suffer hydrolysis above pH 7. Thus, the particles will be degraded into the LAM polymer in different rates according to the different pH's. To prove this point, microparticles were dispersed in D_2O/NaOD (at pH 12), where their dissolution occurred in less than 5 minutes. The result was then analyzed by ^{1}H-NMR (Figure S6), where the signal from the peak of the triazole formed during the Huisgen cycloaddition, was clear to see at 8.39 ppm. This result is a complementary information to the formation of the microparticles through the Cu (I) catalyzed reaction. Furthermore, to understand the cumulative release profile of the microparticles in comparison to their degradability, FITC-dextran (70 kDa) was encapsulated inside the microparticles. The release profile and particle degradability were evaluated in physiological conditions, through the incubation of the particles in PBS at pH 7.4 and at 37 ºC. Additionally, the microparticle cytocompatibility was assessed by live-dead assay and morphology analysis using fluorescence CLSM.

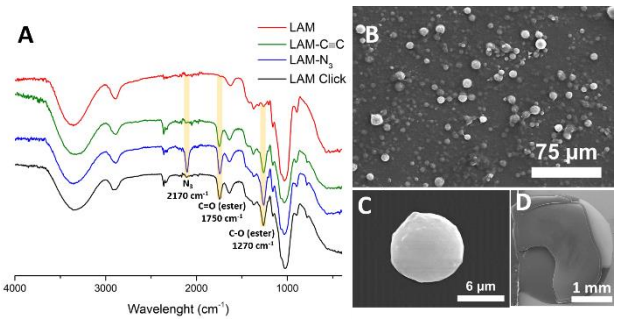


Figure 2 – (A) FTIR analysis of the microparticles precursors. LAM (unmodified); LAM-N_3 (modified with azide); LAM-C≡C (modified with propargyl) and LAM click microparticles, where no vibration corresponding to the azide or propargyl group could be found. (B and C) SEM morphology analysis of the LAM microparticles at two different magnifications. (D) SEM morphology analysis of unmodified LAM.

Release Studies and Particle Biodegradability. In order to evaluate the potential of the microparticles as drug-delivery agents, the release profile was determined. For that, the particles were loaded with FITC-dextran (70 kDa), a high molecular weight polymer coupled to a fluorophore, as a way to prove the high uptake potential of macrosystems, such as proteins. Thus, with an incorporation of 85% of FITC-dextran (70 kDa), the microparticles were left on a heating bath during 11 days in physiological-like conditions (Figure 3A). A fast release during the first day occurred, reaching 41 ± 6% release of the overall cargo loaded, and then a slower release occurs releasing around 10% per day till the fourth day. From there, a slower stabilization occurs, and was followed till the eleventh day, reaching a total of 80 ± 5% of release. This result when compared to the previous published dextran microparticles, that have a similar degree of

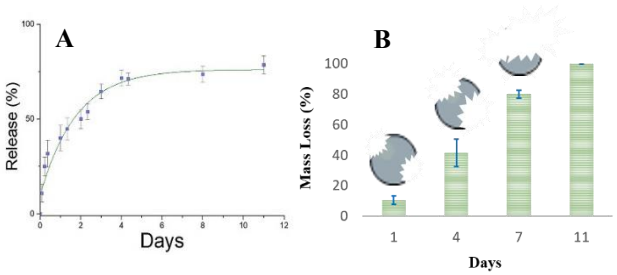


Figure 3 – (A) Cumulative release profile of FITC-dextran from LAM microparticles in PBS, 37 ºCat pH 7.4. (B) Microparticle degradation assessed by mass percentage loss of the microparticles.

substitution, and presented by the same method, seemed to have a release two times faster[18]. Microparticles biodegradability was also performed in physiological-like conditions and followed by 11 days, leading to what may be considered a full degradability of the particles (Figure 3B). The release of the macromolecules seemed to occur prior to the full degradability of the microparticles, due the FITC-dextran diffusion through the pores formed on the first days of degradation. Furthermore, to evaluate the basicity of the different ions presented in the distinct mediums used for cell culture, more assays were conducted in α-MEM and DMEM-LG, in which DMEM has a difference of almost 2-fold of the carbonate ion concentration. All the experiments were conducted during 72 h, at pH 7.4 and in a humidified incubator at 37 ºC, to mimic the physiological conditions (Figure S7). As expected, the higher concentration of carbonate ions (equal to DMEM-LG), triggered the particles degradation, and after 72 h particles were fully degraded. On the other hand, in α-MEM, particles degradation was also clear, but full degradability was still far from accomplished. Moreover, similar results were obtained using the same mediums but without the supplementation with FBS, confirming that the proteins in the serum have no impact on their degradation.

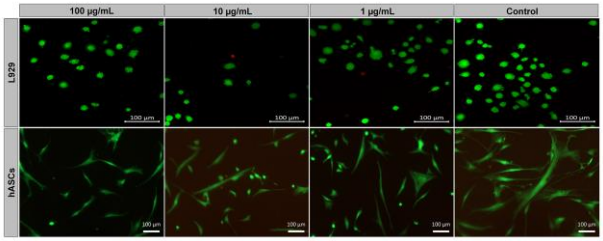


Figure 4 – Live-dead images of L929 fibroblasts and hASCs incubated with different microparticles concentrations and without particles (control) at 24 h. Live cells – green channel (Calcein-AM); dead cells – red channel (PI).

Furthermore, degradation was evaluated in equimolar ratios (to DMEM-LG or α-MEM) of a carbonated aqueous solution. Likewise, to the higher concentration of carbonate ions (equal molarity to DMEM-LG), microparticles full degradability was achieved, while for the lower concentration (equal molarity to α-MEM), the degradation was clear but not complete (Figure S7). This assay was performed to verify the impact of the ionic strength on microparticles degradability since, at the same pH, the two mediums used, differ on the rate of the microparticle degradability. Each medium was chosen due to compatibility to cell line. Mainly, sodium carbonate is the only ionic compound that may have a direct impact on the microparticles degradability and still diverge quite substantially, in quantity, from each medium. Thus, it was shown, that not only the increment of the pH affect the degradability but also the ionic strength of carbonate ions, a basic specie, can interfere.

Cell Viability and Morphology. L929 mouse fibroblasts and hASCs were used to evaluate the cytotoxicity profile of LAM microparticles through live-dead assays. After 24 h (Figure 4) of incubation at 37 ºC, cell viability was analyzed by image acquisition. In order to evaluate the different aspects that could affect the toxicity of the microparticles, cells were also seeded with copper (II) chloride and LAM (Figure S8). Microparticles cytotoxicity, when incubated with L929 or hASCs was similar to that of the control, indicating that cell viability was not affected. Also, microparticle at different concentrations (1, 10 and 100 µg/mL) seemed to have no effect on cell viability (Figure 4). Additionally, the cells incubated in the presence of the LAM polymer did not affect either cell proliferation or viability. In the case of cells incubated with copper, there was a decrease on the viability of both cell types viability when compared with the control. This result led us to two conclusions: first, the copper previously detected onto the microparticles may be coordinated with the polysaccharides[41], and secondly, even after full degradability, the copper that may be released is not sufficient to damage the cells[42]. Therefore, LAM microparticles, from the first results, and up to 72h, which in DMEM-LG medium corresponded to full degradability, seemed to be compatible in both type of cells. As a complementary assay, the morphology

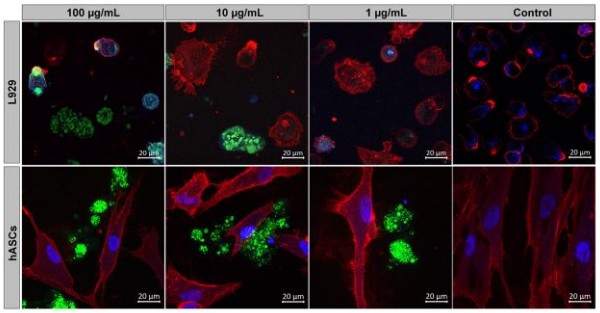


Figure 5 – Fluorescence morphology images of L929 fibroblasts and hASCs after 24h of incubation with different microparticles concentrations. Laminarin microparticles – green channel (FTIC-dextran); Cell membrane – red channel (WGA); Cell nucleus – blue channel (DAPI).

analysis of L929 and hASCs was performed for 24h (Figure 5) and 72 h (Figure S9), where cells' adhesion, spreading and proliferation could be observed. Additionally, microparticles did not suffer uptake (Figure 5), making this type of microcarriers good to reduce the cytotoxicity or enhance hydrophilicity of target specific macromolecules (e.g. tumor specific dendrimers[43]) Furthermore, after 72 h of incubation with L929 fibroblasts, no particles were visible (Figure S9), due to the full degradability in DMEM-LG, and faster dispersion and photobleaching of the FITC-dextran molecules. Lastly, no live-dead assay was performed at 72 h due to proliferation behavior observed in morphology images after 72 h upon incubation (Figure S9).

4. Conclusion

In this work, we reported the synthesis of LAM microparticles stabilized by click chemistry, a bioactive polymer that exhibits relevant properties for biomedical applications. Microparticles were synthesized in mild conditions and evaluated as micro carriers through the potential biodegradability and sustained release in physiological conditions. Moreover, the encapsulation of FITC-dextran (70 kDa), proved the possibility to include macromolecules, such proteins, inside the particles. Furthermore, microparticles were incubated with human adipose stem cells and mouse fibroblasts, where no cytotoxicity was found by evaluation of cell proliferation and viability. Additionally, cancer cells' overexpression of glucose transporters[44, 45] and LAM's potential to induce apoptosis of the colon cancer cells[29-31] may

be an indicative as a new way of treatment. This is expected due to a higher selectivity of the colon cancer cells towards the sub product (LAM polymer–glucose terminals) of the microparticles degradation. Degradability into LAM (Mw < 10 kDa) is likely, as most small linear polymers[46], to be cleared from the organism by the kidneys, thus reducing in-blood circulation time and consequent accumulation issues. Concluding, this type of drug-delivery system may be applied in different fields due to the versatility of applications based on polysaccharides[37, 38].

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