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Abstract: The grafting of galactose units onto poly(-caprolactone) (PCL) substrates by a wet chemistry two-step procedure is proposed. Even though a reduction of hardness from 0.58-0.31 GPa to 0.12–0.05 GPa is achieved, the chemical functionalization does not negatively affect the tensile modulus (332.2 ± 31.3 MPa and 328.5 ± 34.7 MPa for unmodified and surface-modified PCL, respectively) and strength (15.1 ± 1.3 MPa and 14.8 ± 1.5 MPa as assessed before and after the surface modification, respectively), as well as the mechanical behavior evaluated through small punch test. XPS and Enzyme-linked lectin assay (ELLA) demonstrates the presence, and also the correct exposition of the saccharidic epitope on PCL substrates. The introduction of carbohydrate moieties on the PCL surfaces clearly enhances the hydrophilicity of the substrate, as the water contact angle decreases from 82.1± 5.8° to 62.1± 4.2°. Furthermore, biological analysis shows human mesenchymal stem cell viability over time and an improvement of cell adhesion and spreading.

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Milano, 10th of June 2014

Dear Editor,

We are pleased to sumit the following paper to ***Carbohydrate Research***

Manuscript title: Designing smart biomaterials for tissue engineering: carbohydrate functionalized Poly(ε-caprolactone)

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Significance of the manuscript: the paper describes the functionalisation Poly(ε-caprolactone), PCL, with a biologically relevant monosaccharide, in a two-step process downstream to material fabriction. Covalent bonding of bioactive molecules at material surface represents a valid strategy for material functionalisation, since it may permit site-directed immobilization avoiding stochastic biomolecul exposition on the surface. Even though robust techniques for surface "biodecoration" are currently required, the appropriate surface functionalization still remains a critical variable for the optimal performance of a wide range of biomaterials.

Preliminary biological tests showed cell viability at shorter time for surface-modified substrates, while the difference in terms of water contact angles would suggest that the surface treatment should enhance cell density and spreading.

- The manuscript, or its contents in some other form, has not been published previously by any of the authors and/or is not under consideration for publication in another journal at the time of submission

- *All* authors have seen and approved the submission of the manuscript

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Best regards

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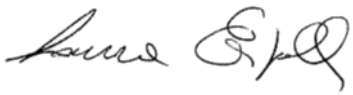

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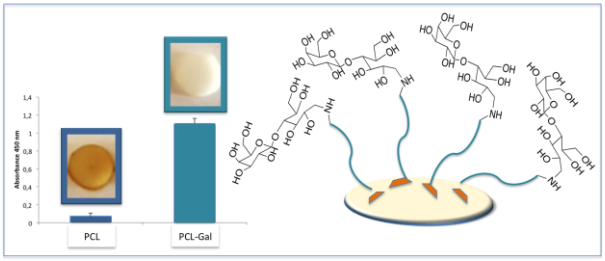
Graphical Abstract



Manuscript title: Designing smart biomaterials for tissue engineering: carbohydrate functionalized Poly(ε-caprolactone) (By Laura Russo et al.)

***Highlights (for review)**

- PCL was functionalized with galactose on its surface
- Reductive amination wss used for PCL functionalization
- ELLA shows correct exposition of sugar moieties
- Human mesenchymal stem cell viability, adhesion and spreading is improved on neoglycosylated PCL

Designing smart biomaterials for tissue engineering: carbohydrate functionalized Poly(ε-caprolactone)

Laura Russo,[a] Teresa Russo,[b] Chiara Battocchio,[c] Francesca Taraballi,[a,d] Antonio Gloria,[b] Ugo D'Amora,[b] Roberto De Santis,[b] Giovanni Polzonetti,[c] Francesco Nicotra,[a] Luigi Ambrosio,[b] Laura Cipolla*[a]

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Abstract. The grafting of galactose units onto poly(ε-caprolactone) (PCL) substrates by a wet chemistry two-step procedure is proposed. Even though a reduction of hardness from 0.58-0.31 GPa to 0.12−0.05 GPa is achieved, the chemical functionalization does not negatively affect the tensile modulus (332.2 ± 31.3 MPa and 328.5 ± 34.7 MPa for unmodified and surface-modified PCL, respectively) and strength (15.1 ± 1.3 MPa and 14.8 ± 1.5 MPa as assessed before and after the surface modification, respectively), as well as the mechanical behavior evaluated through small punch test. XPS and Enzyme-linked lectin assay (ELLA) demonstrates the presence, and also the correct exposition of the saccharidic epitope on PCL substrates. The introduction of carbohydrate moieties on the PCL surfaces clearly enhances the hydrophilicity of the substrate, as the water contact angle decreases from 82.1± 5.8° to 62.1± 4.2°. Furthermore, biological analysis shows human mesenchymal stem cell viability over time and an improvement of cell adhesion and spreading.

Keywords: Poly(ε-caprolactone), carbohydrates, bioactive substrates, tensile properties, small punch test, nanoindentation, biological evaluation.

1. Introduction

Bioactive materials, in which the material scaffold has been functionalized with biomolecules in order to promote the desired biological response, increase the chances of tissue regeneration and wound healing.[1] A valuable way to design new "smart biomaterials"[2] is focused on the amelioration of their bioactivity by the use of adhesive cues (the most widespread approach uses Arg-Gly-Asp peptide sequence to mimic the integrin-signaling domain of fibronectin), targeting

cell adhesion receptors (such as integrin or syndecan)[3], other regulatory molecules (i.e. cytokines, growth factors) and small molecules such as kartogenin (KGN) promoting chondrocyte differentiation for cartilage repair[4] or acetylcholine targeting guanine nucleotide–binding protein (G protein)–coupled receptor used for applications in neural repair.[5] In this scenario, despite their importance, glycans have not been given as much attention as signaling molecules in biomaterial design for tissue engineering and regenerative medicine applications.[6] It is now well established that glycan interactions with their receptors play a fundamental role in various critical intra- and inter-cellular events.[7, 8,] Moreover, carbohydrate structures encode information that modulates interactions between cells, or cells and the ECM.[9, 10] Consequently, these interactions are of high biological relevance[11] and should be included in the investigation of functional biomaterials.

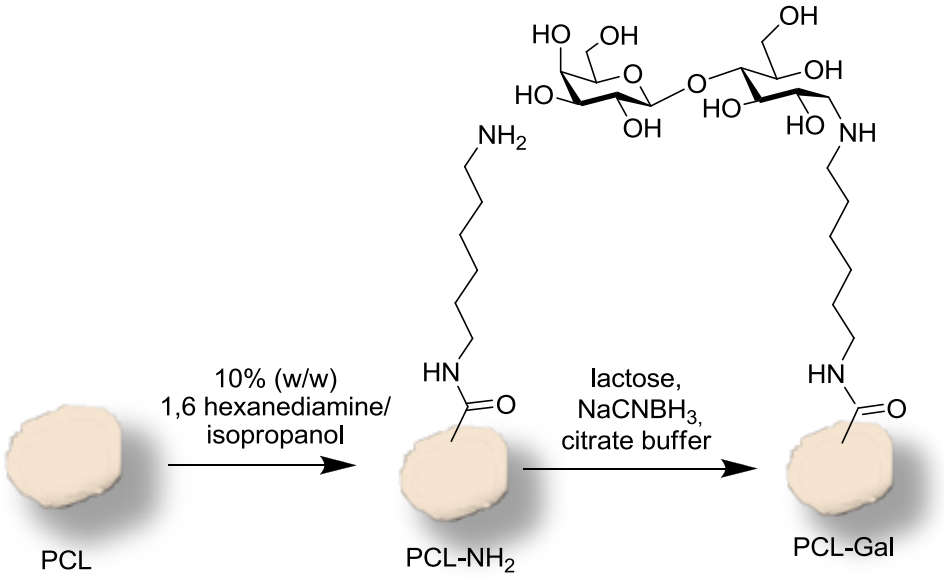
On the basis of these premises, saccharidic motifs are interesting cues to be used for the upgrading of synthetic or natural polymers to smart biomaterials able to cross-talk with their biological environment. Synthetic polymer-based artificial ECMs are generally fabricated from biocompatible, biodegradable polymers to avoid chronic foreign body reactions.[12, 13] Among synthetic polymers, aliphatic polyesters such as poly(ε-caprolactone) (PCL) have been widely considered as biomaterials for designing scaffolds to support the regeneration of several tissue-engineered organs,[14, 15, 16] due to a unique combination of biodegradability and biocompatibility properties. Modification of PCL-based materials is desired in order to improve their hydrophilic properties and to achieve a friendly interface for living cells.[6j,l 17]

Here, we propose the grafting of galactose units onto PCL substrates by a wet chemistry two-step procedure: i) introduction of functional groups (primary amines) by polymer aminolysis[18, 19, 20, 21] and ii) grafting of the saccharidic motif. The chemical composition as well as the hydrophilic

behaviour of the functionalized surface was characterized by X-ray photoelectron spectroscopy (XPS) and water contact angle (WCA) measurements. In order to assess both the efficacy of the chemical procedure and the correct surface exposition of the glycidic cues on PCL surface, recognition assays by commercially available peroxidase- and FITC-conjugated lectins[6k,,22] were used. The effect of the surface modification on the mechanical performances of the substrates at different scales as well as on the biological behaviour *in vitro* was assessed.

2. RESULT AND DISCUSSION

Glycosylated PCL was obtained by covalent conjugation of the carbohydrate onto PCL surface (Scheme 1). Aminolysis of ester bonds of PCL by 1,6-hexanediamine allowed the grafting of amino groups onto the surface of PCL, affording PCL-NH_2 (Scheme 1). The primary amino group was exploited for the reductive amination with the aldehyde group of the reducing end of lactose, thus allowing the grafting of galactose moieties on PCL surface (PCL-Gal, Scheme 1).



Scheme 1. Wet chemistry strategy for PCL functionalization with galactose units.

The quantitative analysis of PCL modified substrates (PCL-NH_2 and PCL-Gal) using the ninhydrin assay shows that NH_2 density is 2.42 μmol/cm^2 on the PCL-NH_2 and 1.89 μmol/cm^2 on the PCL-Gal, indicating that the amount of sugar is roughly 0.50 μmol/cm^2 The presence of monosaccharides introduced by reductive amination reaction on the PCL surface was also investigated by XPS. XPS measurements were carried out at C1s, O1s and N1s core levels on PCL-NH_2 and glycosylated PCL (PCL-Gal) substrates. Pristine PCL was used as control. C1s and N1s spectra collected on the two functionalized samples are reported in Figure 1; Binding Energy (BE), Full Width Half Maximum (FWHM) and atomic ratio values of all core-level signals measured on all samples are collected in Table 1 in the supporting material.

C1s XPS signals of pristine PCL and samples PCL-NH_2 and PCL-Gal are reported in Figure 1a). All spectra appear structured, and the contributions arising by chemically different C atoms were individuated by applying a peak-fitting procedure[23] (coloured curves in Figure 1a). Pristine PCL spectrum contains at least three contributions arising by aliphatic C atoms (C-C, BE = 285.0 eV), C atoms single-bonded to O (C-O, BE = 286.7 eV) and C atoms double-bonded to oxygen in C(O)O groups (BE = 288.9 eV), in excellent agreement with the assignments reported in the literature for this polymer;[24] samples PCL-NH_2 and PCL-Gal C1s spectra are also composited and four contributions arising at very similar BE values, but of different intensities, can be individuated. The intense feature at 285.0 eV is attributed to aliphatic C atoms of the primary amine and PCL substrate; the component at about 286.3 eV is associated with C-N groups, and this feature is not observed in pristine PCL, as expected; the third component at about 287.4 eV is due to C atoms bonded to O through a single bond, i.e. C-O of PCL and C-OH of the saccharide, that are not distinguishable due to the experimental resolution. The last feature, appearing as a peak in pristine PCL and a shoulder of lowering intensity in PCL-NH_2 and PCL-

Gal, is attributed to carboxyl-like carbons.[25,26]It is noteworthy that the two last components are of the same intensity in pristine PCL, as expected from the molecular structure; the intensity of the signal associated with C-O groups considerably increases after glycosylation due to the C-OH contribution, as a consequence of the monosaccharide insertion on the sample surface.

Since PCL does not contain nitrogen atoms, N1s is an utterly indicative signal to probe the reaction successfulness. Furthermore, the nitrogen atom at the top of the primary amine is directly involved in the conjugation with the saccharidic motif. In Figure 1c) N1s XPS rough data collected on samples PCL-NH_2 and PCL-Gal are overimposed, evidencing the presence in the glycosylated sample of a peak at lower BE values that is not observed in PCL-NH_2; as shown in Figure 1b), the peak-fitting allows to individuate a single N1s component in PCL-NH_2 spectrum, associated with N1s atoms of NHCO and $–NH_2$ functional groups (about 400 eV),[27] and two components in PCL-Gal spectrum, due respectively to the new secondary amine N atoms (C-NH-C, at about 398.3 eV) and to the unperturbed NHCO group (about 400 eV).[26] The two peaks observed in PCL-Gal should have theoretically the same intensity (since C-NH-C / NHCO = 1 /1), but the signal at higher BE values arises from N atoms at the interface with PCL, and its photoelectrons are shielded by the functionalization, resulting in lower signal intensity (sampling depth of XPS is about 1000A).

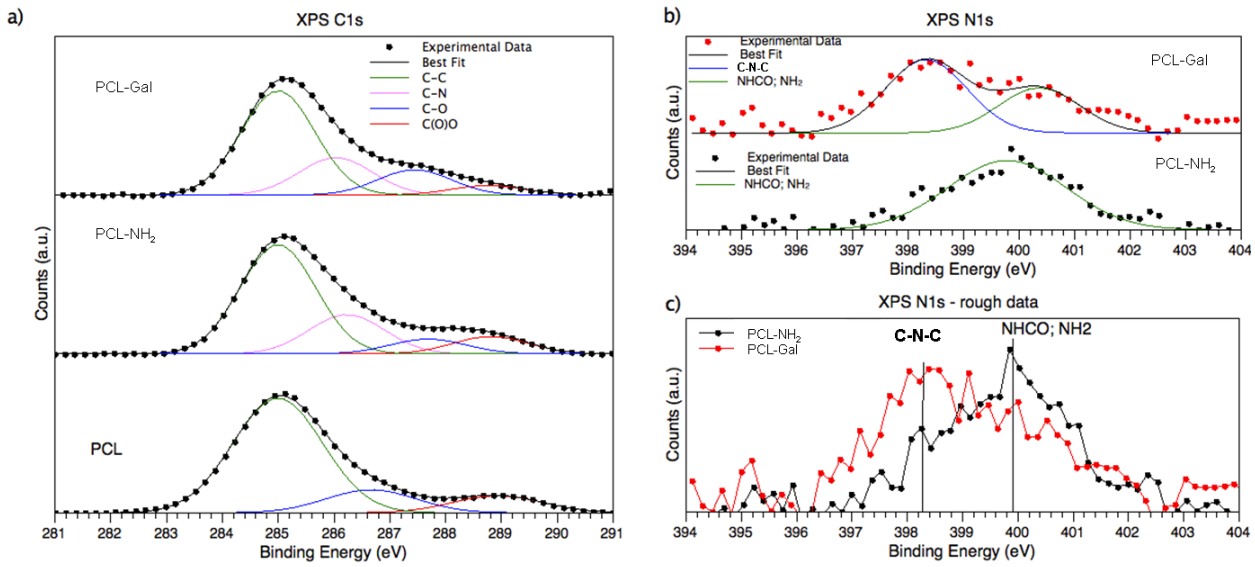


Figure 1. a) C1s XPS spectra collected on pristine PCL (bottom), PCL-NH_2, and PCL-Gal; spectral components are also reported as coloured curves. b) N1s XPS spectra of samples PCL-NH_2 (bottom) and PCL-Gal (top), together with the curve-fitting result (coloured curves). c) Rough XPS data collected at N1s core level on the two functionalized samples, evidencing the C-NH-C contribution appearing as a new peak in PCL-Gal spectrum.

The effect of the surface modification on the mechanical performance of the material was evaluated through classical tensile measurements, small punch and nanoindentation tests. Results from tensile tests have shown a ductile behavior for both unmodified and surface-modified PCL samples. Accordingly, the tensile stress-strain curves were characterized by an initial linear region, then a little decrease in the slope occurred up to a local maximum stress value, followed by a decrease of the stress. A plateau-like region was then evident, and finally a new increase of stress was achieved until failure was generally reached. In particular, the results have highlighted that the surface treatment does not negatively affect the values of tensile modulus (E) and maximum stress (σ_{max}) (Table 1).

On the other hand, the small punch test was chosen to evaluate the mechanical performances since it should be considered as a reproducible miniature specimen test method which has been already employed to assess the mechanical properties of implants retrieved (explanted) from the human body, ultra-high molecular weight polyethylene and acrylic bone cement, as well as of PCL-based substrates reinforced with sol-gel synthesized hybrid microfillers or superparamagnetic iron-doped hydroxyapatite.[28, 29]

Results from small punch tests on unmodified and surface-modified substrates have shown that load-displacement curves are generally characterized by an initial linear region, followed by a decrease of the curve slope until a maximum load was reached. Finally, it was well evident that a decrease of the load until failure had occurred for both kinds of substrates. Values of peak load, representing the initial local maximum in the load-displacement curve, and work to failure, which is the area under the load-displacement curve, are reported as mean ± standard deviation in Table 1.

Materials	**Tensile test**		**Small punch test**	
	E (MPa)	σ_{max} (MPa)	Peak load (N)	Work to failure (mJ)
Unmodified PCL	332.2 ± 31.3	15.1 ± 1.3	29.2 ± 2.0	36.8 ± 7.0
Surface-modified PCL	328.5 ± 34.7	14.8 ± 1.5	28.8 ± 2.4	38.3 ± 7.2

Table 1. Results from tensile and small punch tests performed on unmodified and surface-modified PCL substrates: tensile modulus (E), maximum stress (σ_{max}), peak load and work to failure, reported as mean value ± standard deviation.

Surface treatment does not negatively alter the mechanical behavior under tensile and small punch testing. Wettability and hydrophilicity of the surface-modified PCL substrates were suitably analyzed using water contact angle measurements (Table 2).

Materials	Water Contact Angle θ (°)
Unmodified PCL	82.1 ± 5.8
Surface-modified PCL	62.1 ± 4.2

Table 2. Water contact angles reported as mean value ± standard deviation for unmodified and surface-modified PCL substrates.

As expected, the values of the water contact angle achieved for the surface-modified PCL substrates are lower (62.1 ± 4.2°) than the neat PCL ones (82.1 ± 5.8°) and consistent with those alredy reported for glucosamine-bound PCL (63.0 ± 4.0°).[16]

The water contact angle measured for PCL substrates should be greater than 90° since PCL is a hydrophobic polymer as it is well reported in the literature. However, in contrast to this, Table 2 reports a water contact angle of 82.1 ± 5.8° for unmodified PCL substrates. This result may be ascribed to the specific techniques (melting and moulding) employed to manufacture the substrates that should alter the surface topography and roughness, thus affecting the expected value of the water contact angle. Anyway, this result is also consistent with those reported in previous works on neat PCL substrates obtained through melting and moulding techniques (75.0 ± 5.0°)[6l] or through moulding and solvent casting methods (81.4 ± 4.4°).[28]

In the current work, nanoindentation has been considered to assess the effect of the proposed treatment on the surface properties, as this testing method enhances upon the spatial, force, and displacement resolutions of the traditional indentation techniques, providing a powerful tool to

study biomaterials and natural tissues with submicrometer resolution.[30] This technique should be employed for mapping the surface mechanical properties and for measuring those of microstructural features within bulk samples, as it bridges the gap between atomic force microscopy and macroscale mechanical testing. Nanoindentation measurements on PCL substrates have shown differences in terms of load-depth curves and, hence, of hardness values. Both unmodified and surface-modified PCL substrates have displayed hardness (H) values that generally decrease as load increases from 1 to 5 mN. In particular, measurements on unmodified PCL substrates have provided hardness values spanning from 0.58 to 0.31 GPa in the investigated load range. These values are greater than those obtained for PCL samples which underwent surface modification (0.12−0.05 GPa) (Figure 2a), thus suggesting that after the treatment the surface becomes softer. Consistently with hardness values, the reduced modulus (E_r) of unmodified PCL substrates (4.9−2.6 GPa) is higher than that obtained from the surface-modified ones (1.3−0.5 GPa) (Figure 2b). It is worth nothing that the reduced modulus should be considered a "combined modulus" as it is related to the Young's moduli of both tip and sample, and to their Poisson's ratios. It is well known that values of hardness and modulus are related to polymer chain flexibility and, consequently, to physical and chemical entanglements. The topological restriction of molecular motion by other chains is represented by the density of entanglements among molecular chains, which contribute to the rigidity of amorphous region of the polymers.[31] Accordingly, molecular chains determine the nanoindentation behavior. In the first stage, the aminolysis starts preferentially at the amorphous regions of the polymer and cause the scission of the chains, reducing the density of entanglements.[16, 17] As a consequence of the scission of the chains undergoing functionalization, the mobility and flexibility of molecular chains increases, and the surface of the treated substrates becomes softer.

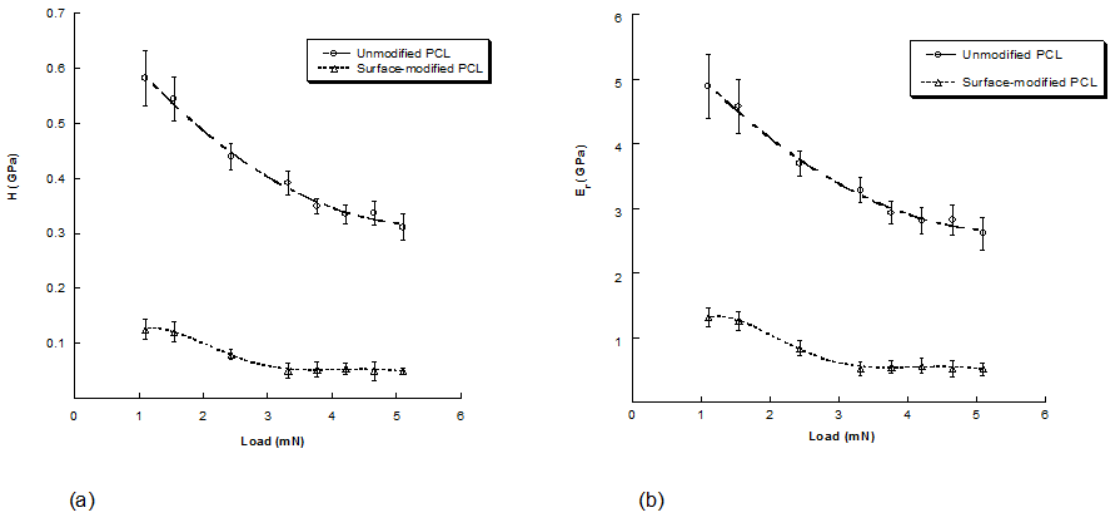


Figure 2. Results obtained from nanoindentation tests on unmodified and PCL-Gal substrates: hardness (a) and reduced modulus (b) as a function of the applied load (1−5 mN). Data are graphically reported as mean value, and bar represents the standard deviation. The dashed lines are a guide for the eye.

Thus, even though the proposed surface treatment locally reduces the hardness of the substrates, it does not negatively affect the tensile modulus and strength, as well as the mechanical behavior assessed through small punch test.

Carbohydrates are able to exploit their biological function only if correctly exposed to their complementary receptors.[60] In order to demonstrate both the functionalisation and at the same time the correct presentation of the galactose unit on the PCL surface, lectin-based assays were used. Lectins are very specific carbohydrate-recognising proteins that are commercially available conjugated to fluorescent probes, as FITC, or peroxidase for Enzyme-linked lectin assay (ELLA).[22] PCL samples, after appropriate blocking to minimize non-specific binding, were incubated with peanut lectin from *Arachys hypogaea* (PNA) labelled with FITC (PNA-FITC) or horse radish peroxidase (PNA-HRP) specific for β-galactosides; after incubation, the samples

were washed to remove excess of lectin. The unmodified PCL samples were used in order to provide a comparison to the neoglycosylated surfaces. Fluorescence analysis of PNA-FITC treated samples are reported in Figure 3. ELLA assay was performed on PNA-HRP-treated films by reaction with soluble peroxidase indicator (*o*-phenylenediamine, OPD). The absorbance of the resulting surnatant, measured at 450 nm (Figure 3) indicates the presence of lectin bound to PCL surface.

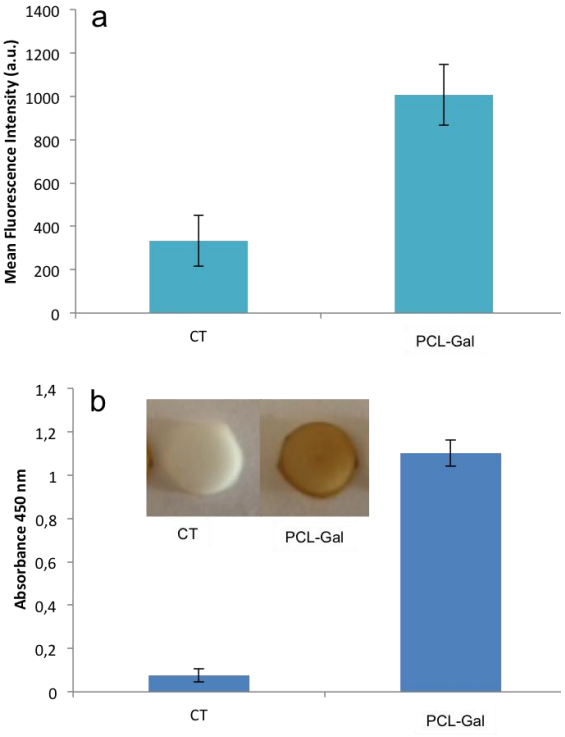


Figure 3. (a) Fluorescence analysis with PNA-FITC; (b) ELLA assay with PNA-HRP conjugate

With regard to the biological performance of the surface-modified substrates, the Alamar Blue assay was performed in order to assess the cell viability. Such assay has provided information on cell viability over time through a quantitative evaluation of the percentage of Alamar Blue reduction at 1, 7, 14 and 21 days. The obtained results are graphically reported as mean value ± standard deviation in Figure 4.

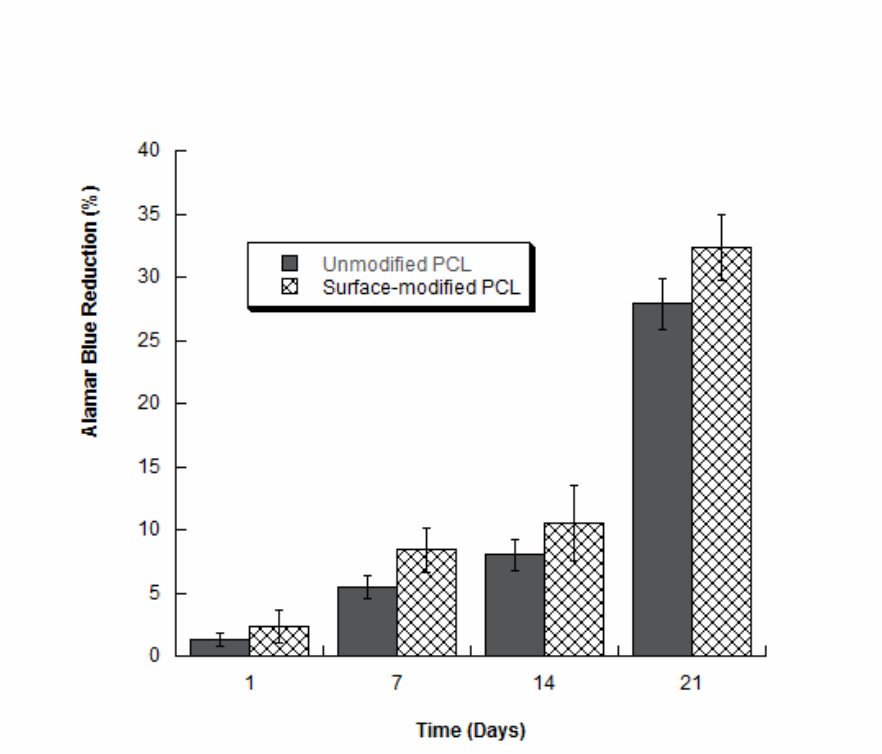


Figure 4. Alamar Blue Assay: results (mean value ± standard deviation) reported at 1, 7, 14 and 21 days.

Figure 4 clearly suggests cell viability over time for both unmodified and surface-modified substrates, as the percentage of Alamar Blue reduction increases with time.

Cell adhesion was then qualitatively investigated through microscopy. In particular, CLSM analysis has shown that hMSCs already adhere on both unmodified and surface-modified substrates at 24 h after seeding, showing a morphology which changes on the different samples (Figure 5).

Results from this analysis have highlighted that cells better adhered on the glycosylated PCL substrates if compared to cells seeded on the unmodified ones.

Specifically, as for the unmodified PCL, cells appear more rounded (Figure 5). On the contrary, with regard to glycosylated PCL substrates, cells adhered and were well spread, indicating a good interaction with the material (Figure 5).

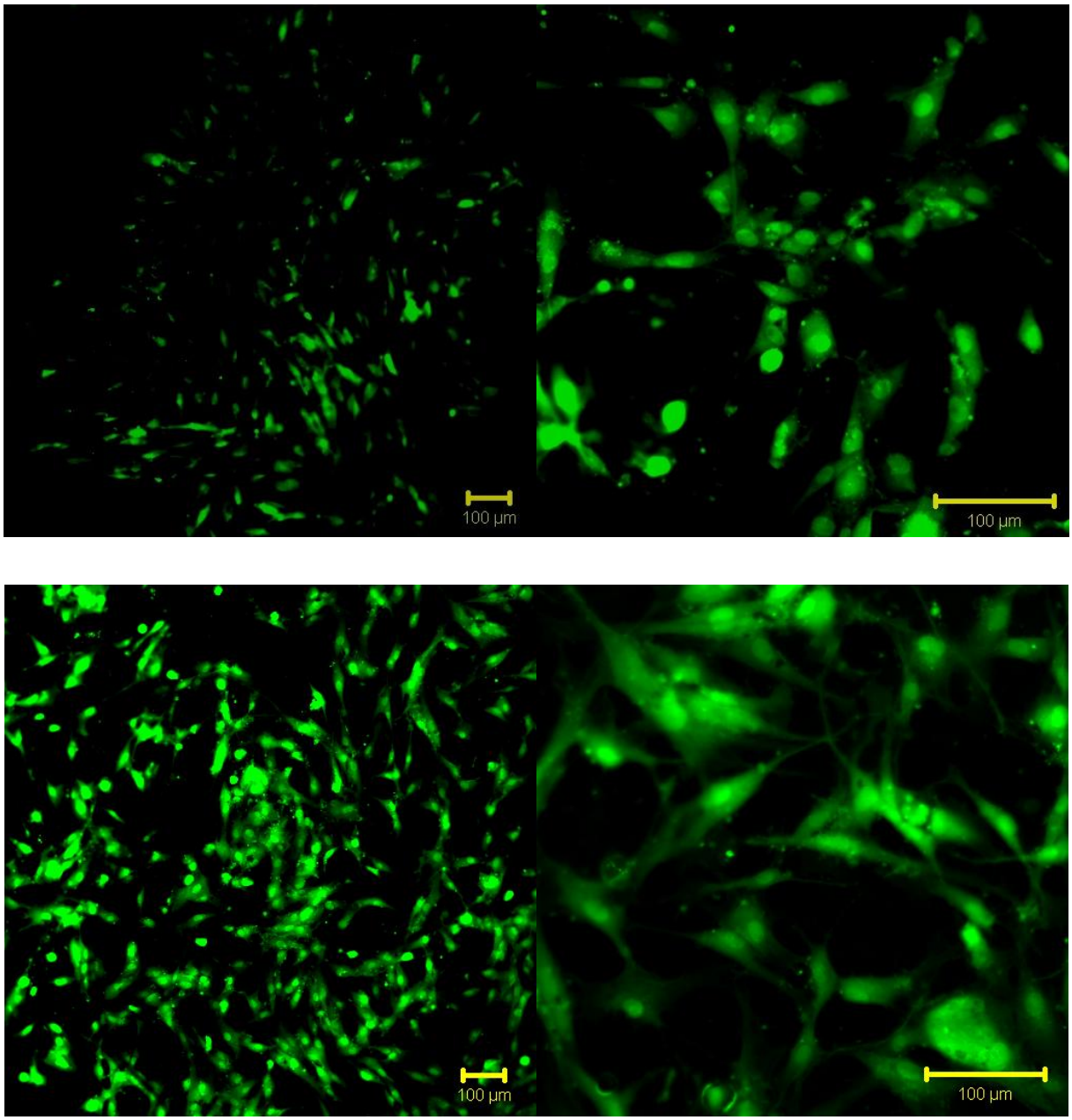


Figure 5. Cell adhesion study - CLSM images at 24 h after cell seeding for unmodified (upper) and surface-modified (lower) PCL substrates: hMSCs were stained with ER tracker green dye and live-cell imaging was performed. Scale bar: 100 µm.

Furthermore, microscopy and Crystal Violet staining have provided information on cell morphology and distribution at longer times (Figure 6), basically confirming the results obtained from CLSM analysis at 24 h after seeding.

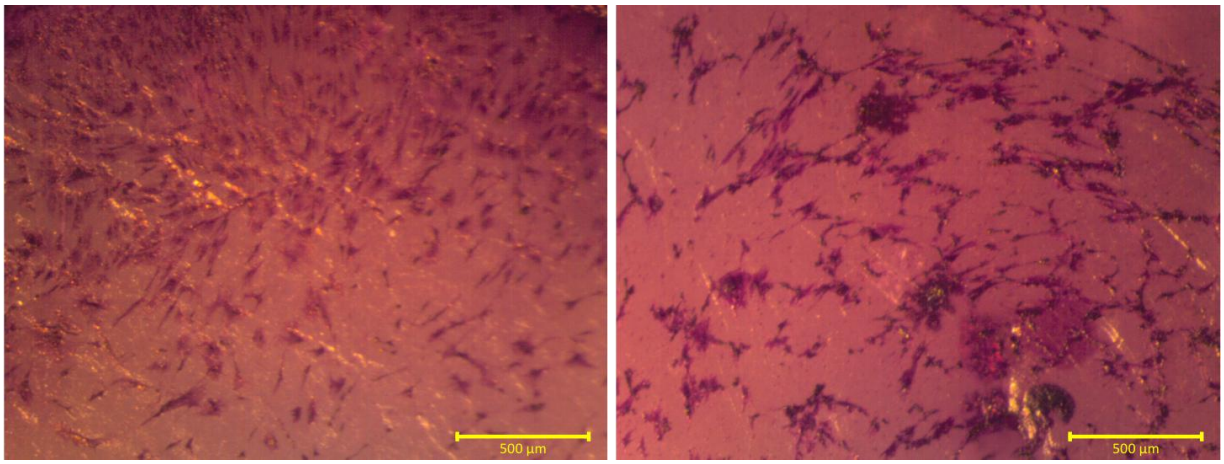


Figure 6. Cell adhesion study - Microscopy and Crystal Violet assay: images of cell-seeded substrates at 21 days after seeding for unomodified (left) and surface-modified (right) PCL substrates. Scale bar: 500 µm.

Figure 6 reports typical images for unmodified and glycosylated PCL cell-seeded substrates after crystal violet staining.

In conclusion we can observe that even though the Alamar Blue assay has demonstrated cell viability over time for both unmodified and surface-modified PCL, CLSM and Crystal Violet staining have evidenced that the grafting of galactose units onto PCL substrates enhances cell density and spreading.

3. Experimental Section

All reagents and all organic solvents were purchased from Sigma Aldrich and used without further purification.

3.1 Substrates preparation. Substrates were basically manufactured through melting and molding technique using PCL (M_w=65000 – Aldrich) pellets. Briefly, PCL pellets were heated at 100°C and the molten polymer was then poured into a suitable mould to obtain standard disk-

shaped (with a diameter of 6.4 mm and a thickness of 0.5 mm) and microtensile specimens, where it was allowed to cool.

3.2 Aminated PCL substrates (PCL-NH_2). The grafting of amino groups onto the surface of PCL by 1,6-hexanediamine (Scheme 1) was performed as already reported.[16] Briefly, aminolysis was conducted by immersing the substrates in a 10% (w/w) 1,6 hexanediamine/isopropanol solution at 37°C with suitable stirring for 30 minutes. After treatment, the samples were rinsed extensively with deionized water. Subsequently, the substrates were dried in a vacuum desiccator at room temperature for 24 h.

3.3 Neoglycosylated PCL (PCL-Gal). Aminated PCL substrates was immersed in citrate buffer (pH 6.00, 1 mL) containing 0.66 mM lactose and 0.33 mM $NaCNBH_3$ and reacted overnight. Then the samples were rinsed extensively with deionized water. Subsequently, the substrates were dried in a vacuum dessicator at room temperature for 24 h.

3.4 Amino and carbohydrate quantification. The amino group surface amount as well as the immobilized carbohydrates on the PCL substrates were measured by the ninhydrin analysis method.[32] The substrates were immersed in 2 mL of 1.0 mol/L ninhydrin/ethanol solution for 1 min and then placed into a glass tube, and heated to 80 °C until complete dissolution of PCL substrates. The obtained solution was diluted to a final volume of 2 mL with ethanol. 0.400 mL of this solution was further diluted to a final volume of 2 mL with ethanol. The absorbance was then recorded with a UV spectrophotometer (Ultrospec 2100 Pro – Amersham Biosciences®) at 560 nm. A calibration curve was obtained using standard solutions of propylamine in ethanol.

3.5 XPS analysis. XPS analysis was performed in an instrument of our own design and construction, consisting of a preparation and an analysis UHV chamber, equipped with a 150 mm mean radius hemispherical electron analyser with a four-elements lens system with a 16-channel

detector giving a total instrumental resolution of 1.0 eV as measured at the Ag $3d_{5/2}$ core level. MgKα non-monochromatised X-ray radiation (hν = 1253.6 eV) was used for acquiring core level spectra of all samples (C1s, N1s and O1s). The spectra were energy referenced to the C1s signal of aliphatic C atoms having a binding energy BE = 285.00 eV. Atomic ratios were calculated from peak intensities by using Scofield's cross section values and calculated λ factors. Swift 24 Curve-fitting analysis of the C1s, N1s and O1s spectra was performed using Gaussian profiles as fitting functions, after subtraction of a Shirley-type background.[33.]

3.6 Tensile Tests. Tensile tests were carried out on both unmodified and surface-modified PCL samples according to the ASTM D1708 standard. The engineering stress (σ) was calculated as follows**:**

$$\sigma = \frac{F}{A_0} \quad (1)$$

where F is the measured force and A_0 represents the cross section area. The engineering strain (ε) was evaluated as the ratio between the elongation (Δl) and the original distance between the upper and lower grips (l_0):

$$\varepsilon = \frac{\Delta l}{l_0} \quad (2)$$

All the tests were performed using an INSTRON 5566 testing machine.

3.7 Small Punch Tests. Small punch tests were performed on unmodified and surface-modified PCL disk-shaped specimens with a diameter of 6.4 mm and a thickness of 0.5 mm, according to the ASTM F2183 standard. Basically, each specimen was loaded axisymmetrically in bending by a hemispherical head punch at a constant displacement rate of 0.5 mm/min until a failure occurred. Peak load and work to failure were evaluated and reported as mean value ± standard deviation.

3.8 Contact Angle Measurements. Contact angle measurements were carried out on unmodified and surface-modified PCL substrates by using a DATAPHYSICS OCA 20 apparatus. Briefly, distilled water was dropped on each specimen in different sites, and the contact angle was measured. Results were reported as mean value ± standard deviation.

3.9 Nanoindentation tests. Nanoindentation tests were carried out on unmodified and surface-modified PCL substrates. Measurements were performed in a specific load range (1−5 mN), using a Nanotest Platform (Micromaterials, U.K.) with a diamond pyramid-shaped Berkovich-type indenter tip. Trapezoidal load functions characterized by a peak load hold period of 20 s and a loading-unloading rate of 300 μN/s were imposed. Load-depth curves, hardness values, and reduced modulus were evaluated. Hardness and reduced modulus were calculated using the Oliver and Pharr method.[34, 35] In particular, hardness (H) was evaluated considering the applied peak load (P_{max}) and the projected contact area (A_c) at the specified load, according to the equation:

$$H = \frac{P_{max}}{A_c} \quad (3)$$

The projected contact area A_c is related to the geometry of the tip and it is evaluated from the penetration depth. Hardness and reduced modulus were reported as mean value ± standard deviation.

3.10 Fluorescence microscopy. All images were acquired using an inverted microscope (Axiovert; Zeiss, Oberkochen, Germany). PCL and neoglycosidated PCL are suspended in a PBS solution of l Lectin from Arachis hypogaea (peanut) FITC conjugated (PNA-FITC, Sigma-Aldrich catalogue n° L7381, 40 μg/ml) and stirred for 2 h in dark conditions. After this time the collagen films were thoroughly washed with PBS buffer (63 times x 15 min). The samples were air-dried and analysed for their fluorescence.

3.11 ELLA assay. PCL and neoglycosylated PCL samples were treated with a solution of 2% BSA in PBS (100 μL) and shaken (14 h, 5°C), according to manufacturer protocol. The samples were then removed and incubated at room temperature with a solution of the lectin from peanut (A*rachis hypogaea*) conjugated to Horse Radish Peroxidase (Sigma-Aldrich, catalogue n° L7759) (0.01 mg/mL, 200 μL) in PBS for 2 h with shaking. The substrates were then thoroughly washed with PBS to remove unbound lectin and then treated with a solution of OPD (SIGMAFASTTM OPD, Sigma-Aldrich, catalogue n°P9187) (500 μL, 1 h). The absorbance of an aliquot of this solution (200 μL) was measured at 450 nm.

3.12 Cellular Assays

Human mesenchymal stem cells (hMSCs, 1 * 10^4 cells per sample) were seeded on unmodified and surface-modified PCL substrates and grown in Dulbecco's modified Eagle's medium (DMEM) without fetal bovine serum (FBS).

The different kinds of cell constructs were analysed at 24 h after cell seeding using confocal laser scanning microscopy (CLSM Zeiss LSM 510/Confocor2) and ER-Tracker™ green for live-cell endoplasmic reticulum labeling. The images of cell constructs were acquired by using a Ar excitation laser at the wavelength of 488 nm and a 10 X objective.

Crystal violet staining and an Olimpus SZX7 stereomicroscope were also employed to assess cell-material interactions at longer times (21 days after cell seeding).

In order to evaluate cell viability/proliferaion, Alamar Blue assay (AbD Serotec Ltd., UK) was also performed on the cell constructs.

Disk-shaped substrates were prepared for cell seeding by soaking first in 70% ethanol for 1 h, then in 1% antibiotic/antimycotic in PBS for 2 h and prewetted in medium for 2 h.

Cells (density of 1 *10^4 cells per sample) were statically seeded onto unmodified and surface-modified substrates.

The Alamar Blue Assay is based on a redox reaction that occurs in the mitochondria of the cells. Thus, the coloured product is transported out of the cell and can be measured through a spectrophotometer. Specifically, at 1, 7, 14 and 21 days after cell seeding, the optical density was measured with a spectrophotometer (Sunrise; Tecan, Männedorf, Zurich, Switzerland) at wavelengths of 570 and 595 nm. The number of viable cells correlates with the magnitude of dye reduction and is expressed as a percentage of Alamar Blue reduction, according to the manufacturer's protocol. Each experiment was performed at least three times in triplicate

4. Conclusion

Despite the relevance of carbohydrates as signaling biomolecules, glycan epitopes are being rarely considered to design biomimetic surfaces, in order to improve cell behaviour at the cell-material interface. Accordingly, cell adhesion should be suitably enhanced and tailored since it represents the basic feature in the cell-material interaction. Previous works have already evidenced that aminolysis represents an easy route to introduce primary amines with high yield that can be easily optimized. Here, we proposed a two-step procedure to immobilize carbohydrate motifs on PCL substrate surfaces. Nanoindentation, tensile and small punch tests allowed the characterization of the effect of the functionalization on the surface and bulk properties. More importantly, the surface modification did not negatively affect the macromechanical behavior of the substrates. Furthermore, the presence of galactose clearly enhances the hydrophilicity of the substrate as demonstrated by the values of the water contact angle, suggesting an improvement of cell behaviour. Biological evaluation has provided

information on cell adhesion and viability, evidencing that the grafting of galactose units onto PCL substrates enhances cell density and spreading. It is worth noting that the functionalization/bioactivation procedure proposed for PCL substrates could be extended to three-dimensional (3D) rapid prototyped scaffolds, thus developing multifunctional porous structures for tissue engineering. Moreover, the proposed methodology may be further extended to the grafting of complex carbohydrates, since it is based on the complementary and chemoselective reaction between the carbonyl group at the reducing end found in mono-, oligo- and polysaccharides and the amino group so easily introduced in polymers. For this reason, the current study may be considered as a first step of a future complex work with the aim of designing 3D scaffolds with controlled architectural features, macro-, micro-, nano-mechanical performances and specific bioactive moieties at the surface.

ASSOCIATED CONTENT

Supporting Information. Full XPS characterization of PCL substrates.

Acknowledgment

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Abbreviations

ECM, Extra Cellular Matrix; ELLA, Enzyme Linked Lectin Assay; OPD, orthophenylendiamine; PBS, Phosphate Buffered Saline; TE, Tissue Engineering.

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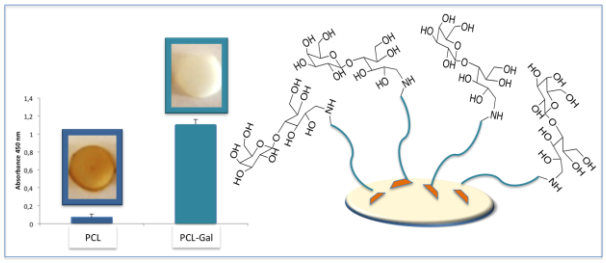
Graphical Abstract



Manuscript title: Designing smart biomaterials for tissue engineering: carbohydrate functionalized Poly(ε-caprolactone) (By Laura Russo et al.)

Designing smart biomaterials for tissue engineering: carbohydrate functionalized Poly(ε-caprolactone)

*Laura Russo, Teresa Russo, Chiara Battocchio, Francesca Taraballi, Antonio Gloria, Ugo D'Amora, Roberto De Santis, Giovanni Polzonetti, Francesco Nicotra, Luigi Ambrosio, Laura Cipolla**

Supporting Information

Table S1. XPS

sample	signal	BE (eV)	FWHM (eV)	atomic percent [a] (%)	attribution
PCL	C1s	285.00	1.89	74.1	C-C
		286.70	1.89	14.8	C-O
		288.94	1.89	11.1	C(O)O
	O1s	532.09	1.84	59.5	C=O
		533.54	1.84	40.5	C-O
PCL-NH_2	C1s	285.00	1.59	59.5	C-C
		286.23	1.59	21.4	C-N
		287.49	1.59	9.7	C-O
		288.80	1.59	9.4	C(O)O
	O1s	531.58	1.80	44.2	C=O
		532.69	1.80	40.0	C-O
		533.77	1.80	15.8	H_2O
	N1s	399.80	2.21	100	-NHCO (amide-like) + -NH_2
PCL-Gal	C1s	285.00	1.55	59.1	C-C
		286.04	1.55	21.1	C-N

	287.45	1.55	14.3	C-O
	288.77	1.55	5.5	C(O)O
O1s	531.05	1.69	58.8	C=O
	532.23	1.69	32.8	C-O
	533.54	1.69	8.4	H_2O
N1s	398.33	1.72	62.0	C-N-C
	400.36	1.72	38.0	-NHCO (amide-like) + $-NH_2$