Poly(*N*-Isopropylacrylamide) Surface-Grafted Chitosan Membranes as a New Substrate for Cell Sheet Engineering and Manipulation

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ABSTRACT: The immobilization of poly(*N*-isopropylacrylamide) (PNIPAAm) on chitosan membranes was performed in order to render membranes with thermo-responsive surface properties. The aim was to create membranes suitable for cell culture and in which confluent cell sheets can be recovered by simply lowering the temperature. The chitosan membranes were immersed in a solution of the monomer that was polymerized via radical initiation. The composition of the polymerization reaction solvent, which was a mixture of a chitosan non-solvent (isopropanol) and a solvent (water), provided a tight control over the chitosan membranes swelling capability. The different swelling ratio, obtained at different solvent composition of the reaction mixture, drives simultaneously the monomer solubility and diffusion into the polymeric matrix, the polymerization reaction rate, as well as the eventual chain transfer to the side substituents of the pyranosyl groups of chitosan. A combined analysis of the modified membranes chemistry by proton nuclear magnetic resonance (^{1}H-NMR), Fourier transform spectroscopy with attenuated total reflection (FTIR-ATR) and X-ray photoelectron spectroscopy (XPS) showed that it was possible to control the chitosan modification yield and depth in the solvent composition range between 75% and 100% of isopropanol. Plasma treatment was also applied to the original chitosan membranes in order to improve cell adhesion and proliferation. Chitosan membranes, which had been previously subjected to oxygen plasma treatment, were then modified by means of the previously described methodology. A human fetal lung fibroblast cell line was cultured until confluence on the plasma-treated thermo-responsive chitosan membranes and cell sheets were harvested lowering the temperature.

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Introduction

Poly(*N*-isopropylacrylamide) (PNIPAAm) is a soluble polymer in cold water that present a sudden precipitation upon heating above the lower critical solution temperature (LCST), at around 32°C in pure water (Fujishige et al., 1989; Kubota et al., 1990; Scarpa et al., 1967). This transition involves the breakage of intermolecular hydrogen bonds with the water molecules, which are replaced by intramolecular hydrogen bonds amongst the dehydrated amide groups. Subsequently, the PNIPAAm molecules assume a globule conformation, exposing the hydrophobic isopropyl groups to the water interface (Baysal and Karasz 2003; Graziano 2000). Therefore, when immobilized onto a solid substrate, the LCST behavior of PNIPAAm render the surface with thermo-responsive wettability (da Silva et al., 2007). The temperature control over the wettability of the PNIPAAm-grafted substrates has been used to modulate protein adsorption (Duracher et al., 2004; Huber et al., 2003; Yamato et al., 2000) and confluent cells cultured on such substrates can be recovered as a contiguous cell sheet just by lowering the temperature (da Silva et al., 2007; Kikuchi and Okano, 2005; Shimizu et al., 2003; Yang et al., 2005). In

conventional cell culture on tissue culture polystyrene (TCPS), cells are harvested disaggregating the extracellular matrix (ECM) through the enzymatic action of trypsin and by simultaneously chelating the Ca^{2+} and Mg^{2+} ions with ethylenediaminetetraacetic acid (EDTA). However, non-specific proteases may damage critical cell surface proteins, such as ion channels and receptors, which constitute a major drawback of this cell harvesting method (Canavan et al., 2005; Ide et al., 2006; Nakajima et al., 2001; Von Recum et al., 1999; Yamada et al., 1990). Besides that, the recovery of the cells, together with the intact newly deposited ECM, represents an increased therapeutic potential with respect to the same single cells harvested by the conventional proteolytic methods, unable to keep cells confluence.

The so-called cell sheet engineering has been mainly performed using TCPS dishes grafted with PNIPAAm (da Silva et al., 2007), which are rigid non-swollen supports. The confluent cell sheets cultured in these thermo-responsive substrates present relatively long-detachment times. Several attempts have been made to accelerate the thermal harvesting process, such as grafting PNIPAAm onto porous supports (Kwon et al., 2000) and co-grafting PNIPAAm with poly(ethylene glycol) (PEG) (Kwon et al., 2003). Another noteworthy approach was based on a multi-functional linear block copolymer, poly(N-isopropylacry-lamide-co-acrylic acid)-b-poly(L-lactic acid). In this approach small amounts of acrylic acid were used to accelerate the detachment and the poly(L-latic acid) block assured that the copolymer could be easily processed in non-conventional shapes (Kim et al., 2005).

The potential of cell sheet engineering in the regenerative medicine field has been fostered by the development of manipulation techniques which allow transferring the fragile cell sheets from the thermo-responsive culture substrate to the desired place (Harimoto et al., 2002; Shimizu et al., 2001; Yamato et al., 2001). Single cell sheets has been transplanted directly to human patients for cornea regeneration tissues (Nishida et al., 2004), but they can also be layered in order to recreate thicker tissue-like constructs with homotypic (Shimizu et al., 2002) or heterotypic (Harimoto et al., 2002) cells. However, the number of cell sheets that can be effectively layered without core ischemia or hypoxia is limited, because of restrictions on the delivery of nutrients and accumulation of metabolic wastes (Yang et al., 2005). The thermo-responsive surfaces fabricated to harvest intact cell sheets can also be used to keep the multilayered cell sheets in culture for a certain period of time and to allow the thermal recovery of the thicker constructs. It should be noticed that the thermo-responsive PNIPAAm-grafted TCPS substrates commonly used to culture single cell sheets are impermeable, which would decrease the rate of elimination of metabolic wastes and of nutrients supplying, since the construct side facing the culture surface would be wasted as a potential mass transfer area.

In this work, we propose a methodology to grafted PNIPAAm on chitosan membranes aiming at being used as novel substrates for cell sheet engineering and manipulation.

Chitosan is the soluble derivative of chitin obtained by N-deacetylation, which biocompatibility and non-toxicity make it an excellent candidate as a raw material in the biomedical field (Kumar, 2000). Chitosan has been proposed for a range of controlled drug release formulations (Prabaharan and Mano, 2005; Wang et al., 2007), as rate controlling membranes in transdermal delivery systems (Siddaramaiah et al., 2006; Thacharodi and Panduranga Rao, 1996), as a biomaterial (Lopez-Perez et al., 2007; Silva et al., 2004a,b) and for tissue engineering (Baran et al., 2004; Mano and Reis, 2007; Patel et al., 2007; Silva et al., 2007; Tuzlakoglu et al., 2004). We previously developed chitosan membranes that possess adequate permeation properties for the rapid elimination or delivery of small molecules (da Silva et al., accepted). The use of these membranes grafted with PNIPAAm, if able to functioning as substrate for the thermal recovery of confluent cell sheets, would increase the mass transfer area for nutrients and metabolic wastes, hopefully supporting the culture of thicker layered cell sheet constructs. It has been shown that primary human oral mucosa epithelial cells create stratified epithelial layers when cultured on submicron porous thermo-responsive supports, even if cultured without 3T3 feeder layers and fetal bovine serum (Murakami et al., 2006a,b). Murakami el al. proposed that these results might be explained by the exchange of metabolites, which can occur from beneath the basal layer of the cultured epithelium. In this way, the culture conditions mimic more closely the in vivo state.

Moreover, the PNIPAAm-grafted chitosan membranes reported herein will also be useful to transfer the cell sheets directly to the host site with minimal manipulation. Finally, fully hydrated chitosan membranes should be easily adaptable to several anatomical shapes, owning to its mechanical flexibility (Silva et al., 2004b).

Materials and Methods

Chitosan Material and Other Reagents

Chitosan raw-material from crab shells were purchased from Sigma–Aldrich (St. Louis, MO) and purified prior to use. Chitosan was dissolved at ∼1% (w/v) in an aqueous acetic acid solution (1% w/v). The solution was filtered to remove the insoluble material. The clear solution obtained was precipitated adding a NaOH solution to form a white gel, which was sieved to remove the exuded liquid. This gel was thoroughly washed with distilled water (until no changes on the pH were detected), further washed/dehydrated with ethanol, freeze-dried, ground to powder and dried at 60°C overnight. N-Isopropylacrylamide (NIPAAm) (Acros-Organic, Morris Plains, NJ) was recrystallized from a n-hexane/diethyl ether (5:1) mixture and dried overnight to remove residual solvent. All other reagents were used without further purification.

The chitosan average molecular weight was of found to be 660 kDa by viscometry in CH_3COOH 0.5 M/$NaCH_3COO$

0.2 M, according to the Mark-Houwing theory ($k = 3.5 \times 10^{-4}$; $a = 0.76$) (Terbojevich et al., 1996). The degree of N-deacetylation (DD) was found to be 65.4% by means of first derivative UV spectrophotometry, using both glucosamine (GluN) and N-acetylglucosamine (GluNAc) standards for calibration (da Silva et al., 2008).

Preparation of Chitosan Membranes by Solvent Casting

The chitosan solution was prepared by dissolving chitosan (1 wt.%) in acetic acid solution (1 wt.%). The solutions were carefully stirred in order to avoid the formation of any air bubble, poured on Petri dishes (5 mg of chitosan/cm^2) and dried at room temperature in a dust free environment. The resultant membranes (thickness approximately 50 μm) were neutralized in NaOH 0.1 M solution for 10 min and washed thoroughly with distilled water. The obtained membranes (CTS) were hold in a frame and dried again, presenting a smooth surface without the typical wrinkles derived from the material shrinking during the drying process.

Swelling of Chitosan Membranes in Mixtures of Isopropanol and Water

The swelling of chitosan membranes in mixtures of a non-solvent (isopropanol) and a solvent (water) was determined by immersing previously weighted chitosan membranes in mixtures of these solvents at compositions varying from pure water to pure isopropanol. After around 2 h, equilibrated samples were blotted with filter paper to remove the adsorbed solvent and weighted immediately. The equilibrium swelling ratio (S_{eq}) was calculated using the following equation:

$$S_{eq} (\%) = \frac{W - W_0}{W_0} \times 100 \qquad (1)$$

where W_0 is the initial weight of the sample and W is the weight of the swelled sample.

Surface Modification by Plasma Treatment

In one of our previous works (Lopez-Perez et al., 2007), plasma treatment of chitosan membranes was very effective on improving the viability and proliferation of osteoblast-like cells. In this sense, the surface of some chitosan membranes was modified by plasma treatment according to that procedure (Lopez-Perez et al., 2007), before PNIPAAm grafting. Briefly, the plasma treatment was performed using a radio frequency (13.56 MHz) Plasma Prep5 equipment from Gala Instrument. Samples were exposed to O$_2$ plasma at 30 W of power during 15 min. The pressure in the reactor was maintained under 20 Pa by regulating the gas flow. The samples were only further processed after 48 h in order to assure that free radicals formed during the plasma treatment have been quenched.

PNIPAAm Grafting Onto and Into Chitosan Membranes

The monomer (NIPAAm) was dissolved in several compositions of these isopropanol/water mixtures varying in the volume ratio from (50:50) to pure isopropanol (100:0). The initiator, 2,2′-azobis-isobutyronitrile (AIBN), was dissolved in each solvent mixture used in the respective monomer solution. Chitosan membranes were immersed in the monomer solutions. Both monomer and initiator solutions were deoxygenated under slow nitrogen flow for 10 min. The polymerization was initiated adding the AIBN solution to the monomer solutions and the reaction was performed at 60°C under N$_2$ atmosphere for 18 h. The volumes of NIPAAM and AIBN solutions give a final monomer concentration of 0.25 g/mL and AIBN to NIPAAm molar ratio of 1%. The grafted membranes were washed thoroughly with water/acetone (25:75) to remove un-reacted monomer and unbound polymer. Samples were labeled as iPrOH100, iPrOH90, iPrOH75 and iPrOH50, according to the volume of isopropanol used in the non-solvent/solvent mixture composition. The same PNIPAAm grafting procedure was also applied to plasma-treated chitosan membranes at an isopropanol/water composition of 75:25 (P-iPrOH75).

Assessment of Chitosan Membranes Chemical Modification

PNIPAAm-grafted membranes were analyzed by Fourier Transform Infrared spectroscopy with the attenuated total reflection (FTIR-ATR) to assess the existence of major chemical changes occurring at lower depth. Spectra were recorded in a Perkin-Elmer (Waltham, USA) Spectrum One spectrophotometer (32 scans, resolution 4 cm^{-1}). Proton nuclear magnetic resonance (^{1}H-NMR) was used to estimate the total amount of grafted PNIPAAm, which were thought to be detectable for samples in which grafting reaction was performed in solvents with higher water content. Around 10 mg of each membrane sample was dissolved in 1 mL of 0.4% (w/v) of deuterium chloride (DCl) in D$_2$O solution at room temperature. PNIPAAm spectrum was obtained dissolving in D$_2$O. The ^{1}H-NMR spectra were acquired in a VARIAN INOVA-300 (300 MHz) spectrometer.

X-Ray Photoelectron Spectroscopy (XPS)

Possible chemical changes occurred on the surfaces after the modification were evaluated by XPS. The spectra were obtained using an ESCALAB 200A instrument from VG Scientific (UK) with PISCES software for data acquisition and analysis. A monochromatic Al Kα radiation ($h\nu = 1486.60$ eV) operating at 15 kV (300 W) was used. The measurements were performed in a constant analyzer energy mode (CAE) and take off angle of 90° relative to the sample

surfaces. Survey spectra were acquire using a pass energy of 50 eV, over a binding energy range of 0–1,100 eV, and were used to calculate the elemental composition of the surfaces. Element atomic percentages were calculated from the integrated intensities of the survey spectra using the sensitivity factor of the instrument data system. High-resolution spectra for different regions (C 1s, O 1s and N 1s) were obtained using a pass energy of 20 eV and were peak-fitted using a least-squares peak analysis software, XPSPEAK version 4.1, using the Gaussian/Lorenzian sum function. Background counts were subtracted using a linear baseline and the sample charging was corrected assigning a binding energy of 285.0 eV to the saturated hydrocarbons C 1s peak.

Water Uptake Kinetics and Equilibrium Hydration Degree

The water uptake measurements were undertaken in phosphate buffer saline (PBS) solution, prepared dissolving PBS tablets (Sigma Chemical Co., St. Louis, MO) in a suitable amount of water (NaCl 0.137 M; KCl 0.0022 M; phosphate buffer 0.01 M; pH 7.4 at 25°C). The water uptake was determined immersing previously weighted chitosan membranes in buffer solution at $37 \pm 1°C$. After each time period samples were blotted with filter paper to remove the adsorbed water and weighted immediately. The calculation of the water uptake (WU) was also based on equation (1). The equilibrium hydration degree (WU_{eq}) in PBS solution was taken as the last point of the water sorption kinetic curves.

Cell Culture

A human fetal lung cell line (MRC-5), an immortalized cell line with fibroblast-like morphology, was obtained from European Collection of Cell Cultures (ECACC, UK) and was used in the cell culture studies. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, Inc.) supplemented with 10,000 U/mL penicillin-G sodium, 10,000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B in a 0.85% saline (Gibco, Invitrogen Corporation, Paisley, UK) and 10% of heat-inactivated fetal bovine serum (FBS; Biochrom AG, Berlin, Germany) in a humidified atmosphere with 5% of CO_2 at 37°C. Membranes were cut with 14 mm diameter and placed onto 24-well culture plates. Prior to culturing, all samples were sterilized by adding 1 mL of 70% ethanol aqueous solution for 90 min and subsequently washed with sterile phosphate buffered saline solution (PBS, Sigma Chemical Co.) to remove the remaining ethanol. Cells were seeded on the materials at a concentration of 7×10^4 cells/mL, 1 mL per well and incubated for 10 days, time at which the cells seeded on plasma-treated materials (P-iPrOH75) were 100% confluent.

Cell Sheet Detachment and Assessment of the Cell Viability

After 10 days of culture, plates were removed from the incubator and observed by light microscopy. The cells

cultured on the different samples were continuously observed to assess the eventual detachment from the surface at room temperature (ca. 16°C). Cell viability was assessed after Calcein AM staining. A 2:1,000 Calcein AM solution was prepared with DMEM culture medium and 1,000 μL were added to each sample culture. Plates were incubated for 15 min at 37°C in a humidified atmosphere of 5% CO_2 and cell fluorescence examined in an Axioplan Imager Z1 from Zeiss (Göttingen, Germany).

Results and Discussion

Isopropanol–Water Mixtures Solvent Uptake

The control of the swelling capability of the chitosan membranes can be tightly achieved by changing the composition of isopropanol and water mixtures. As it can be observed in Figure 1, the swelling equilibrium degree varies linearly with the volume composition of the solvent mixtures for concentrations of isopropanol higher than 10%. The swelling decreases steadily with increasing proportions of isopropanol. On the other hand, the membranes did not swell at all for the pure non-solvent. This result should allow engineering membrane surfaces with different grafting yields and depths, by changing simultaneously the monomer uptake by the membranes (solubility), diffusion into the polymer matrix and the monomer reaction rate on the surface or inside the swollen membranes. Of course, the solvent composition is restricted to the isopropanol volume ratio (higher than 50%) in which monomer and initiator are soluble at the polymerization temperature (60°C).

Assessment of Chitosan Membranes Chemical Modification

The FTIR spectrum of PNIPAAm presents two intense bands at 1,650 and 1,544 cm^{-1} (Fig. 2a), which are related to
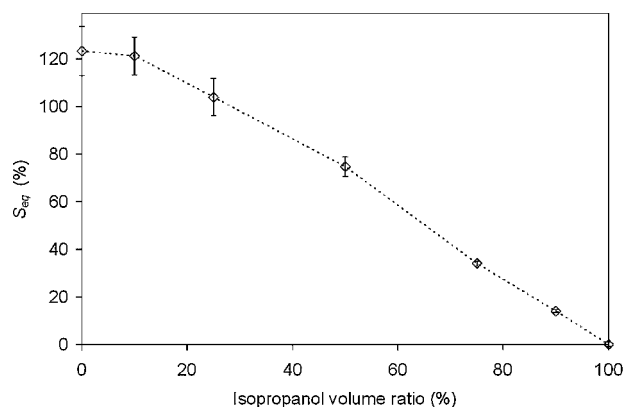
Figure 1. The equilibrium swelling ratio (S_{eq}) of chitosan membranes in mixtures of isopropanol and water at varying compositions represented in function of the isopropanol volume ratio.
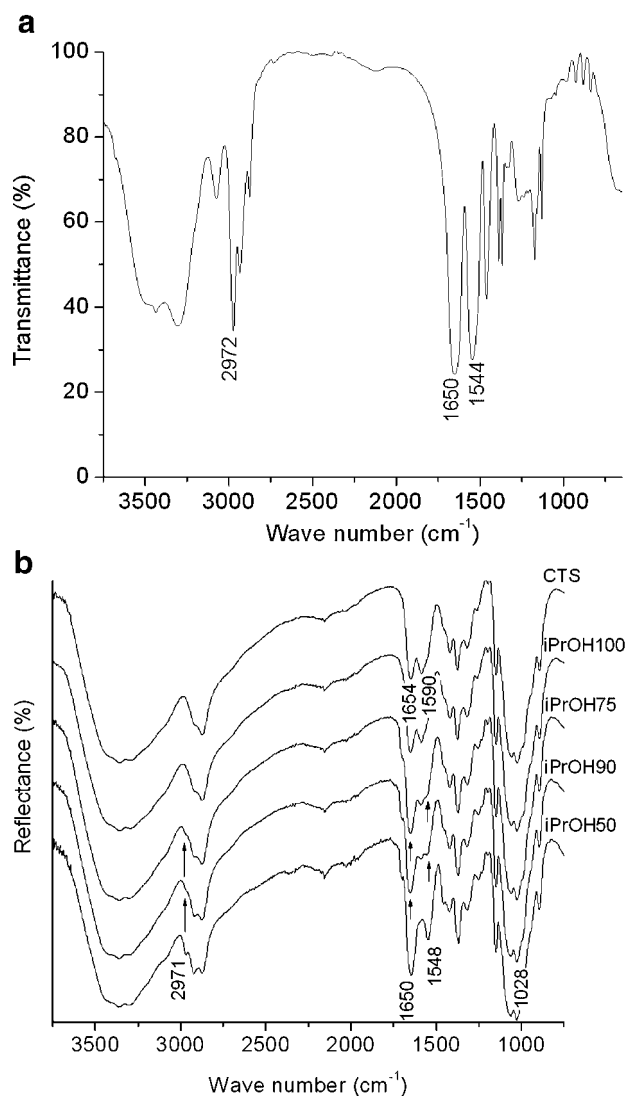
Figure 2. (a) FTIR-ATR spectrum of PNIPAAm; (b) comparative spectra of non-grafted chitosan membranes (CTS) and grafted in mixtures of isopropanol and water at varying compositions (iPrOH100, iPrOH90, iPrOH75 and iPrOH50).

the amide groups. The first is assigned to the stretching vibration of C=O group (amide I), whereas the second corresponds to the bending vibration of NH and the symmetric stretching of N–C=O (amide II). The three bands appearing at 2,972, 2,932, and 2,874 cm^{-1} can be assigned to the stretching vibration of the C–H bonds from the isopropyl groups and polymer backbone. The band at 2,972 cm^{-1} is particularly intense.

In the chitosan membranes (CTS) spectrum (Fig. 2b), amide II band is observed at 1,590 cm^{-1}. The intensity of the band at 1,645 cm^{-1}, which is assigned to stretching vibration of carbonyl group (amide I), is in good agreement with the low deacetylation degree of the used chitosan. The bands assigned to the stretching vibration of C–O–C linkages in the

polysaccharide structure appear at 1,151, 1,060, 1,027, and 895 cm^{-1}. The weak bands at 2,927 and 2,874 cm^{-1} correspond to the stretching vibration of the C–H bonds.

The non-swollen membranes modified in pure isopropanol exhibit an infrared spectrum very similar to the non-modified membranes (CTS) (Fig. 2b). Nevertheless, it does not mean that the surface has not been modified, because in the FTIR-ATR technique the penetration depth of the IR radiation beam is around 1–5 μm into the polymer membrane at each internal reflection (Ratner, 1996). Therefore, information cannot be inferred if the modification only affects the topmost layers of the sample, as it would be expected for the non-swollen membranes.

In the membrane samples modified in an isopropanol content higher than 90% a shoulder or band appears at the amide II region between 1,590 and 1,548 cm^{-1} (see Fig. 2b), which becomes more intense as the solvent swelling ratio increases (from 100% to 50% of isopropanol), indicating a gradual increase in the amount of PNIPAAm. In fact, in the spectrum of iPrOH50 the amide II band suffered a displacement to 1,548 cm^{-1}, which is consistent with the wave number of that band in PNIPAAm. It is also observed an increase on the intensities of the amide I band that can be explained due to the cumulative contribution of PNIPAAm amide groups (C=O) and of the chitosan acetyl groups (C=O). Furthermore, the relative intensity of the characteristic chitosan C–H stretching bands at 2,874 and 2,927 cm^{-1} changed gradually and a new band appears at 2,972 cm^{-1} (iPrOH50), which corresponds to the particularly intense C–H stretching band in the PNIPAAm spectrum. The described spectral changes point out an increase in the amount of PNIPAAm in a superficial region of the membranes (1–5 μm) when going from iPrOH90 to iPrOH50, i.e., when increasing the polymerization solvent swelling ratio.

The ^{1}H-NMR spectroscopy results (Fig. 3) give quantitative information with respect to the bulk modification yield. PNIPAAm pure homopolymer (see Fig. 4) was previously dissolved in D$_2$O and the proton chemical shifts (δ) were assigned: δ 1.00 (–CH$_3$, isopropyl group), 1.42 (–CH$_2$–, polymer chain), 1.78 (–CH–, polymer chain), and 3.76 (–CH–, isopropyl group). Only the first two peaks are well resolved from the chitosan spectrum. On the other hand, the chitosan peak assigned to the H2 proton (δ 2.95) of the GluN units is the only peak well resolved both from the PNIPAAm spectrum and from the HOD signal.

We used the PNIPAAm peak from the isopropyl –CH$_3$ groups (six protons) to estimate the amount of this polymer relative to the amount of chitosan, which in turn was estimated using the GluN H2 proton signal and the DD value. It is clear from Figure 3 that PNIPAAm is undetectable for the samples treated under solvent conditions less favorable for swelling (iPrOH90 and iPrOH100). This means that the modification for low and non-swelling conditions, respectively, is much more superficial, as it would be expected. In turn, the relative amount of PNIPAAm is considerable high for iPrOH75 (5.3 wt.%)
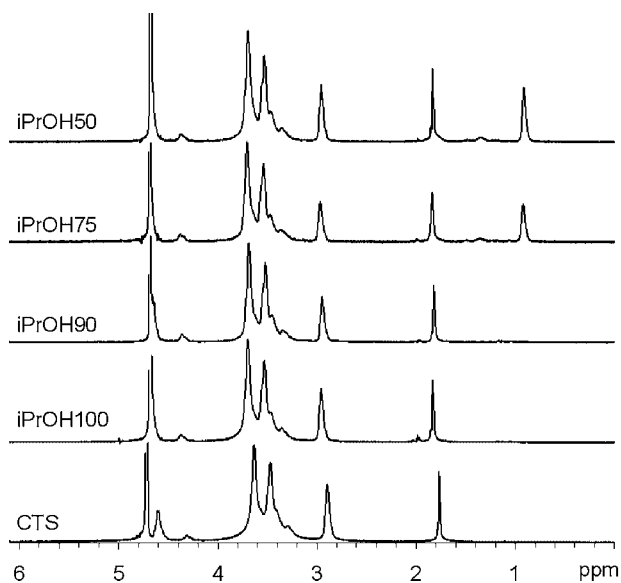
Figure 3. ¹H-NMR spectra of non-grafted chitosan membranes (CTS) and grafted in mixtures of isopropanol and water at varying compositions (iPrOH100, iPrOH90, iPrOH75 and iPrOH50).

and only increases a little for iPrOH50 (5.5 wt.%), indicating a much higher modification depth of these membranes.

Surface Analysis by X-Ray Photoelectron Spectroscopy (XPS)

The surface chemical composition of modified (iPrOH75 and iPrOH100) and non-modified membranes (CTS) was evaluated by XPS analysis. Table I shows the elemental composition of the surfaces (at.%) extracted from the survey spectra. Carbon, oxygen and nitrogen appeared as major components for all the samples, as it was expected considering the chemical structure of chitosan and PNIPAAm (Fig. 4). Some impurities (Ca, Cl, and Zn) were also found, in very low percentage (<0.5%) in the composition of some of the samples and were not considered for the element analysis. After the grafting, a polymer chain (–CH₂–CH–) is introduced on the membranes surface and as it was expected, the C 1s percentage increased in the modified samples compared with the untreated one.
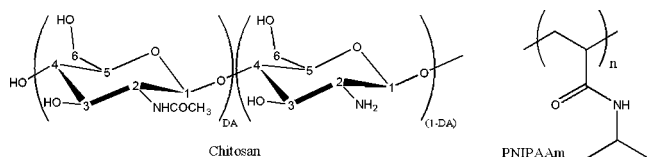

Figure 4. Chemical structures of chitosan and PNIPAAm.

Table I. Elemental composition (at.%) of untreated chitosan membranes (CTS) and modified materials (iPrOH75 and iPrOH100) calculated from the XPS survey spectra.

Element	CTS	iPrOH75	iPrOH100
C 1s (at.%)	65.1	77.5	74.3
O 1s (at.%)	29.2	21.0	22.5
N 1s (at.%)	5.7	1.5	3.2

Figure 5a shows the binding energy region corresponding to C 1s peak (279.8–191.9 eV) for CTS samples. The peak-fitting was performed according with the chitosan chemical structure. The peak at 285.0 eV was assigned to C–H and C–C chemical bonds of the chitosan backbone. The second peak at 286.4 eV corresponded to C–OH, C–O and C–N–C=O carbons and, finally, the peak centered at 287.6 eV was assigned to O–C–O and N–C=O from the acetylated rings. It was not possible to perform the deconvolution of the band corresponding to C–NH₂ bond, because amines are reported to induce small chemical shifts (around 0.6 eV) (Briggs, 1998) and the band should be superimposed by the band of the hydrocarbons chemical bonds in chitosan, observed at 285.0 eV.

In the case of linear PNIPAAm, it is expected a C 1s spectra containing four peaks, allocated in the same position that chitosan peaks. Peaks at 285.0 eV and around 285.4 eV should be associated to hydrocarbons in the polymeric
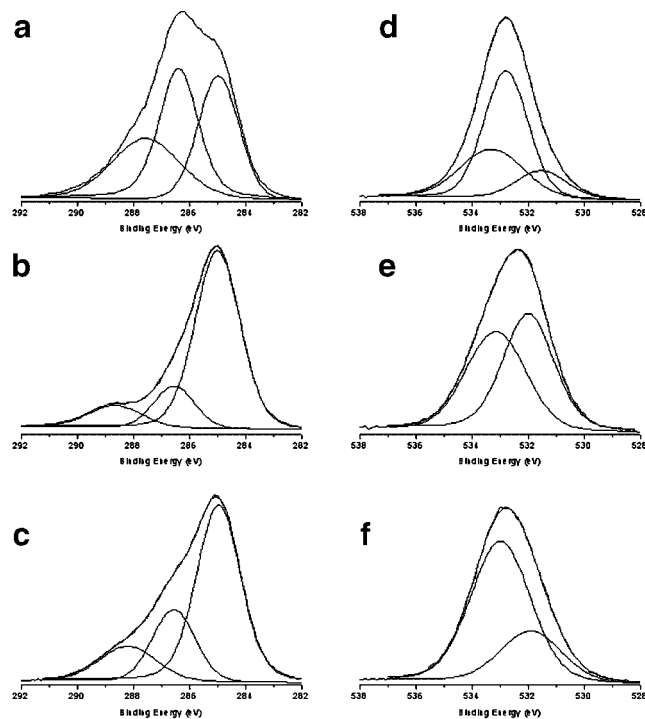
Figure 5. XPS high-resolution spectra for C 1s (left) and O 1s (right). From top to down: CTS, iPrOH75 and iPrOH100.

Table II. C 1s and O 1s core levels composition (%) for CTS, iPrOH75 and iPrOH100.

C 1s core level				O 1s core level			
Bond	CTS	iPrOH75	iPrOH100	Bond	CTS	iPrOH75	iPrOH100
C–C; C–H	33.0	75.3	63.4	N–C=O	14.0	52.8	27.0
C–O; C–N–C=O	38.8	14.8	22.4	C–OH	56.6	47.2	73.1
N–C=O; O–C–O	28.3	9.9	14.1	O–C–O	29.4	0.0	0.0

backbone. The peak corresponding to C–N–C=O should be situated around 286.5 eV and the carbon from the carbonyl group (N–C=O) should be allocated around 288.0 eV (Adem et al., 2005; Bullett et al., 2006).

Figure 5b and c shows the C 1s region for the samples iPrOH75 and iPrOH100, respectively. The intensity of the band corresponding to the hydrocarbon bonds increases in both cases compared with CTS (Table II), providing evidence that PNIPAAm polymer chains have been introduced on the surface through the modification procedure. In the case of the sample iPrOH100, this result had not been possible to confirm using less surface sensitive techniques such as FTIR-ATR, showing that the PNIPAAm chains grafting occurred at a very superficial level (the FTIR-ATR penetration depth of the FTIR-ATR analysis is in the range of 1–5 μm (Ratner, 1996)).

The binding energy region corresponding to O 1s peak (394.0–404.0 eV) for chitosan is showed in Figure 5d. The peak at 531.5 eV was assigned to carbonyl oxygen (N–C=O) presented in the N-acetyl-glucosamine rings. Oxygen atoms involved in hydroxyl bonds (C–OH) were included in the peak appearing at 532.8 eV. The peak at 533.3 eV was identified as characteristic of the O–C–O bonds.

PNIPAAm only posses one oxygen atom per polymer molecule been involved in amide bonds (N–C=O). Therefore, after the grafting, it was expected an increase in the peak allocated around 531.5 eV relatively to the peaks contained single C–O bonds. As can be observed from values showed in Table II, when the modification is performed using isopropanol at 75% (Fig. 5e) the first peak became much more intense, corroborating the PNIPAAm grafting. On the other hand, when 100% isopropanol was used for the grafting reaction (Fig. 5f), this increase was not so drastic showing that the grafting occurred but at lower extent.

Chitosan Membranes Equilibrium Hydration Degree

The chitosan membranes water uptake kinetics (Fig. 6) in PBS solution was very fast, the equilibrium hydration degree (WU_{eq}) being achieved in less than 5 min. It is interesting to notice that the non-modified chitosan membranes (CTS) presented the higher WU_{eq} of approximately 160% and that it decreases from iPrOH100 to iPrOH75, being lower for membranes polymerized at higher swelling solvent conditions. During the NIPAAm polymerization the growing polymer chains predictably occupy the spaces created by the

swelling in the reaction solvent. Being so, the conditions that induce higher swelling rates should allow for the entrance of higher amounts of newly formed polymer. Interestingly, the WU_{eq} is inversely related to the amount of PNIPAAm chains that entered the chitosan membranes pre-established molecular network.

On the other hand, the iPrOH50 equilibrium hydration degree was higher than that of iPrOH75, oppositely to what would be expected. Nevertheless, although the monomer and initiator are both soluble at the polymerization conditions of isopropanol at 50% and 60°C, the polymerized NIPAAm precipitates partially, which may explain such discontinuity in the tendency of equilibrium hydration degree. The precipitation of the newly formed PNIPAAm also explains why the bulk grafting yield calculated by ^{1}H-NMR is quite similar for both iPrOH50 and iPrOH75 samples, although the swelling of the chitosan membranes in isopropanol at 50% is twice than that determined at 75%.

PNIPAAm Covalent Grafting Versus Chain Entanglement

The immobilization of PNIPAAm chains on the membranes may take place by means of covalent bonds, formed by transfer reactions of the growing PNIPAAm radicals to the side substituents of the pyranosyl groups of chitosan. In radical polymerization, the growth of each radical proceeds by successive addiction of monomer units until interrupted
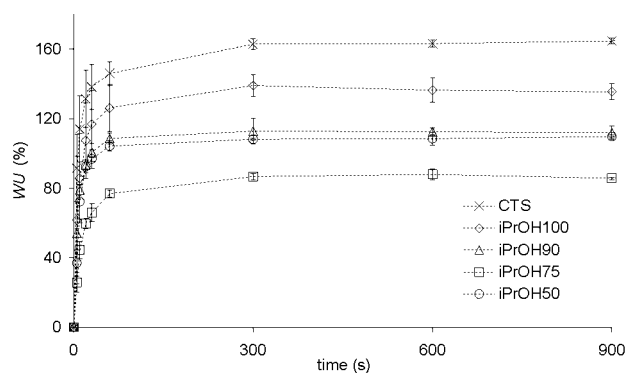

Figure 6. Water uptake kinetics in PBS solution (37°C) of non-grafted chitosan membranes (CTS) and grafted in mixtures of isopropanol and water at varying compositions (iPrOH100, iPrOH90, iPrOH75 and iPrOH50). Data represents mean ± standard deviation for $n = 3$.

by one of the chain termination mechanisms, such as for instance chain transfer. Chain transfer reactions may occur towards the solvents, initiator, monomers, impurities or other molecules present in the reaction system, in an extent that depends on the chain transfer constant of each individual component, according to the Mayo equation (Mayo, 1943). It has been reported that grafting of poly(vinyl acetate) to poly(vinyl alcohol) occurs through a chain transfer mechanism (Okaya et al., 2004). Moreover, it was found that poly(acrylic acid-*co*-acrylamide) chains may be grafted onto starch-based polysaccharides by transfer reactions of the growing radicals on the side substituents of the pyranosyl cycles (Elvira et al., 2002). In fact, chain transfer seems to be an important mechanism on the grafting of PNIPAAm onto chitosan membranes. The XPS core level spectra for C 1s and O 1s show that PNIPAAm is grafted on the surface of non-swollen chitosan membranes (iPrOH100), in which chain entanglement is not an expected mechanism for PNIPAAm chains immobilization. In fact, the non-swollen nature of these samples (iPrOH100) should not allow the penetration of the growing radical and the samples were thoroughly washed with water/acetone to remove the non-grafted PNIPAAm. In the other samples (iPrOH50, iPrOH75, P-iPrOH75 and iPrOH90), polymer chains physical entanglement cannot be discharged as a possible mechanism for PNIPAAm immobilization. Nevertheless, according to the Mayo theory (Mayo, 1943), grafting through chain transfer reaction should be favored inside the membrane, because chitosan (chain transfer agent) concentration is much higher in the interior of the swollen members than at the surface level, oppositely to the solvent concentration, which is higher at the membranes surface.

Cell Sheet Detachment and Assessment of the Cell Viability

The non-modified chitosan membranes (CTS) showed poor cell adhesion and proliferation (see Fig. 7). The few viable cells adhered on the CTS sample surface after 10 days of culture did not present the typical fibroblast-like morphology, being quite round and sparsely distributed. The poor cell adhesion and proliferation on chitosan membranes have been previously reported (Lopez-Perez et al., 2007; Zhu et al., 2005). The iPrOH50 membranes presented very similar results (not shown). In turn, the cells cultured on the membranes modified using solvent compositions varying from 75% to 100% of isopropanol presented the typical elongated fibroblastic morphology. Moreover, it was possible to observe regions on the surface of these samples where cells reach the confluence, but also regions with only few adhered cells. Although the modification with PNIPAAm improved the surface properties in terms of cell behavior, they did not present adequate cell proliferation to reach confluence all over the samples surface, within the predetermined culture period. As our aim is to be able to create
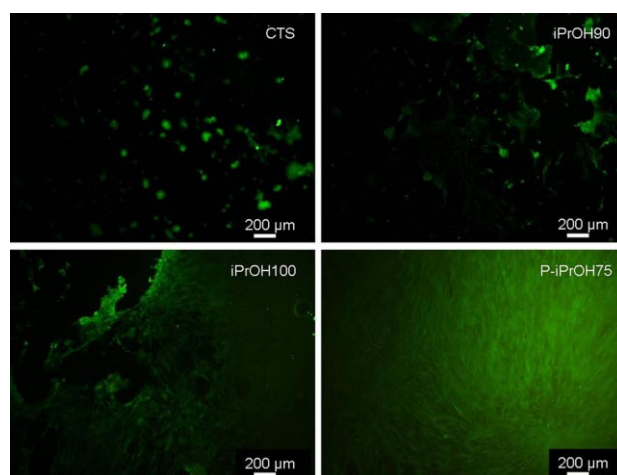
Figure 7. Fluorescence microscopy of viable human fetal lung fibroblast cells stained with calcein AM solution in DMEM culture medium and after 10 days of culture on both non-modified chitosan membranes (CTS) and PNIPAAm grafted (iPrOH90, P-iPrOH75 and iPrOH100). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

cell sheets, which can be harvested by simply lowering the temperature, cell confluence is a critical parameter to achieve. In one of our previous works (Lopez-Perez et al., 2007), plasma treatment was very effective on improving the viability and proliferation of osteoblast-like cells. It is well-known that the surface energy has a striking effect on the cell behavior. We were able to modify chitosan membranes surface by using a suitable oxygen plasma treatment, improving the cell viability and proliferation, which was correlated with both moderate polar and Lewis base components for the surface energy.

In this sense, in this work we used the same PNIPAAm grafting procedure on plasma-treated chitosan membranes (P-iPrOH75). Fully confluent and viable cell sheets were formed on this sample after 10 days of culture (see Fig. 7). As it can be observed in Figure 8, the confluent cell sheets were harvested from the modified thermo-responsive chitosan membranes (P-iPrOH75) keeping the cultured cells at room temperature (ca. 16°C). Although it was not possible to reach 100% confluence on the other PNIPAAm modified chitosan membranes (iPrOH50, iPrOH75, iPrOH90 and iPrOH100), we were able to observe the low temperature detachment of some single cells and smaller patches of confluent cells.

Conclusions

The control of the swelling capability of the chitosan membranes could be tightly achieved by changing the composition of isopropanol and water mixtures, providing a suitable mean to tailor the modification yield and depth. The changes in the FTIR-ATR spectra point out to an increase in
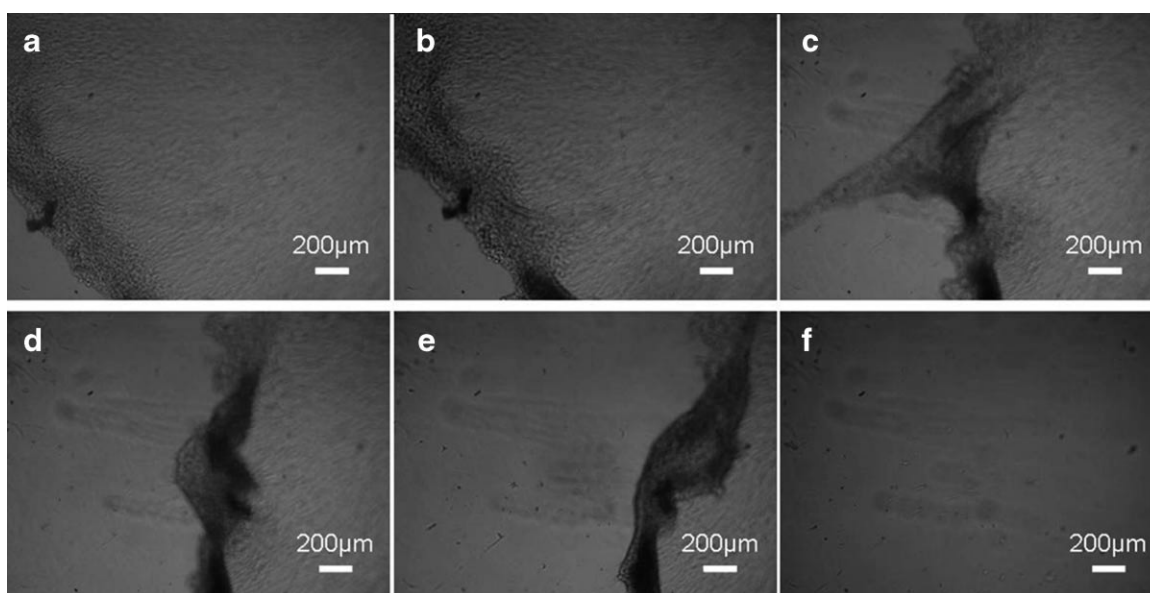
Figure 8. Light microscopy sequence (**a–f**) of the detachment, at room temperature (ca. 16°C), of a confluent cell sheet grown on the PNIPAAm-grafted chitosan membranes (P-iPrOH75).

the amount of PNIPAAm in a superficial region of the membranes (1–5 µm) by means of increasing the polymerization solvent swelling ratio. PNIPAAm was not detected in the ^{1}H-NMR spectra of the samples modified at low (iPrOH90) and non-swelling conditions (iPrOH100), revealing that the modification occurs mainly at superficial level, as it would be expected. In turn, the grafting yield calculated by ^{1}H-NMR is considerable high for iPrOH75 (5.3 wt.%) and iPrOH50 (5.5 wt.%), indicating a much deeper modification of these membranes. The XPS core level spectra for C 1s and O 1s show that PNIPAAm is grafted on the surface of non-swollen chitosan membranes (iPrOH100), in which chain entanglement is not an expected mechanism for PNIPAAm chains immobilization.

The immobilization of PNIPAAm chains on these membranes should take place by transfer reactions of the growing PNIPAAm radicals to the side substituents of the pyranosyl groups of chitosan. In the other samples (iPrOH50, iPrOH75, P-iPrOH75 and iPrOH90), polymer chains physical entanglement cannot be discharged as a concomitant mechanism for PNIPAAm immobilization.

The plasma-treated chitosan membranes grafted with PNIPAAm (P-iPrOH75) produced fully confluent viable cell sheets after 10 days of culture. The confluent cell sheets were harvested from the thermo-responsive chitosan membranes (P-iPrOH75) by lowering the temperature. Although it was not possible to reach 100% confluence on the other PNIPAAm modified chitosan membranes, we were able to observe the low temperature detachment of some single cells and smaller patches of confluent cells.

The use of chitosan membranes, which possess adequate permeation properties for the rapid elimination or delivery of small molecules (da Silva et al., accepted), would increase the mass transfer of nutrients and metabolic wastes, hopefully supporting the culture of thicker layered cell sheet constructs. Finally, fully hydrated chitosan membranes are flexible (Silva et al., 2004b) and they should be easily adaptable to several anatomical shapes, facilitating the transfer of either single cell sheets or layered cell sheet constructs directly to the host site with minimal manipulation.

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