The published version of the paper " W. Yang, E. Fortunati, F. Bertoglio, J. S. Owczarek, G. Bruni, M. Kozanecki, J. M. Kenny, L. Torre, L. Visai, D. Puglia (2018). Polyvinyl alcohol/chitosan hydrogels with enhanced antioxidant and antibacterial properties induced by lignin nanoparticles. Carbohydrate Polymers, 181, 275-284" is available at: https://doi.org/10.1016/j.carbpol.2017.10.084

Polyvinyl alcohol/chitosan hydrogels with antioxidant and antibacterial properties induced by lignin nanoparticles

W. Yang[1], E. Fortunati[1], F. Bertoglio[3,4,5], J. S. Owczarek[2], G. Bruni[6], M. Kozanecki[2], J. M. Kenny[1], L. Torre[1], L. Visai[3,5]*, D. Puglia[1]*

[1] *University of Perugia, Civil and Environmental Engineering Department, Materials Engineering Center, UdR INSTM, Terni – Italy*

[2.] *Lodz University of Technology, Department of Molecular Physics, Lodz - Poland*

[3]*Molecular Medicine Department (DMM), Center for Health Technologies (CHT),UdR INSTM, University of Pavia, Via Taramelli 3/B, 27100 Pavia, Italy*

[4]*Scuola Universitaria Superiore IUSS, Palazzo del Broletto-Piazza della Vittoria, 15, 27100 Pavia, Italy*

[5]*Department of Occupational Medicine, Toxicology and Environmental Risks, Istituti Clinici Scientifici Maugeri S.p.A., IRCCS, Via S. Boezio, 28, 27100, Pavia, Italy*

[6]*Department of Chemistry, — Physical-Chemistry Section, University of Pavia, Viale Taramelli 16, 27100, Pavia, Italy*

Weijun Yang, weijun.yang2012@gmail.com

Elena, Fortunati, elena.fortunati@unipg.it

Federico Bertoglio, federico.bertoglio01@ateneopv.it

Jan Owczarek, 164040@edu.p.lodz.pl

Giovanna Bruni, giovanna.bruni@unipv.it

Marcin Kozanecki, marcin.kozanecki@p.lodz.pl

Josè Maria Kenny, jose.kenny@unipg.it

Luigi Torre, luigi.torre@unipg.it

Livia Visai, livia.visai@unipv.it

Debora Puglia, debora.puglia@unipg.it

Novel polyvinyl alcohol/chitosan hydrogels with antioxidant and antibacterial properties induced by lignin nanoparticles

W. Yang[1], E. Fortunati[1], F. Bertoglio[3,4,5], J. S. Owczarek[2], G. Bruni[6], M. Kozanecki[2], J. M. Kenny[1], L. Torre[1], L. Visai[3,5]*, D. Puglia[1]*

[1] *University of Perugia, Civil and Environmental Engineering Department, Materials Engineering Center, UdR INSTM, Terni – Italy*

[2.] *Lodz University of Technology, Department of Molecular Physics, Lodz - Poland*

[3]*Molecular Medicine Department (DMM), Center for Health Technologies (CHT),UdR INSTM, University of Pavia, Via Taramelli 3/B, 27100 Pavia, Italy*

[4]*Scuola Universitaria Superiore IUSS, Palazzo del Broletto-Piazza della Vittoria, 15, 27100 Pavia, Italy*

[5]*Department of Occupational Medicine, Toxicology and Environmental Risks, Istituti Clinici Scientifici Maugeri S.p.A., IRCCS, Via S. Boezio, 28, 27100, Pavia, Italy*

[6]*Department of Chemistry, — Physical-Chemistry Section, University of Pavia, Viale Taramelli 16, 27100, Pavia, Italy*

**Corresponding authors:*

debora.puglia@unipg.it, Tel +39 0744 492916; Fax +39 0744 492950

livia.visai@unipv.it, Tel +39 0382 987725; Fax: +39 0382 423108

Abstract

Polyvinyl alcohol/chitosan (PVA/CH) hydrogels containing 1 and 3 wt % of lignin nanoparticles (LNP) were prepared through a freezing-thawing procedure. Results from microstructural, thermal and mechanical characterization of LNP based PVA/CH demonstrated that the lowest amount of LNP (1 wt %) was beneficial, whereas the presence of agglomerates at higher LNP content limited the effect. Moreover, a different swelling behaviour was observed for hydrogels containing LNP with respect of PVA/CH, due to the formation of a porous honeycomb-like structure. A synergic effect of CH and LNP was revealed in terms of antioxidative response by DPPH activity of migrated substances, whereas results from antimicrobial tests confirmed LNP as effective against Gram negative bacteria (*E.Coli*) when compared to Gram positive *(S.aureus* and *S. epidermidis)* strains. The obtained results suggested the possible use of produced PVA/CH hydrogels incorporating LNP in many different sectors, such as drug delivery, food packaging, wound dressing.

Keywords: polyvinyl alcohol, chitosan, lignin nanoparticles, hydrogels, antioxidative, antibacterial

1. Introduction

Hydrogels have unique properties, as flexible approach in the synthesis phase, tuneable physical performance, desirable constituents and high biocompatibility. Specifically, their excellent biocompatibility with the human tissue constructs and low irritation to the surrounding tissues make them more attractive in different applications, as in the case of controlled drug and protein delivery, tissue engineering and cosmeceutical (Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009). However, the poor mechanical properties of hydrogels after swelling limited its applications. In order to improve the mechanical properties of hydrogels, some methods has been taken into consideration, like physical blending, chemical modification by grafting, realization of interpenetrating polymer networks, and crosslinking method (J. M. Yang, Su, & Yang, 2004). Polyvinyl alcohol (PVA), a water soluble and semicrystalline plastic, has been widely utilized in preparation of hydrogels, because of its excellent properties, including solvent resistance, mechanical performance, high hydrophilicity and biocompatibility (Kubo & Kadla, 2003; Liu, Chen, Liu, Bai, & Wang, 2014). In addition, it can be easily mixed with some other polymers already widely considered for applications in the cited sectors, one of them is the chitosan (CH), a non-toxic cationic polysaccharide isolated from some natural crustacean shells, like crab and shrimp. CH is a copolymer of 2-glucosamine and N-acetyl-2-glucosamine units, wherein the former constitutes a major fraction of the biopolymer chain. Chitosan has been widely studied for biosensors, tissue engineering, separation film, water treatment, due to its preferable properties as antioxidant, antibacterial agent, and for its good biocompatibility and biodegradability (Crini & Badot, 2008; Kumar, 2000; Rinaudo, 2006). However, some disadvantages such as dissolution in highly acidic solution, low surface area, high cost, poor thermal and mechanical properties restrict its applications. To make up for the advantages and disadvantages of both PVA and CH, hydrogels based on PVA/CH blends have been developed. Nowadays, PVA/CH blend hydrogels have been reported to be used for the controlled release of drugs, because of their low toxicity and high biocompatibility. Kim et al. (S. J. Kim, Park, & Kim, 2003) reported about PVA/CH hydrogels prepared by UV irradiation, which exhibited high dependence of swelling on pH and temperature. Abdel-Mohsen et al. (Abdel-Mohsen, Aly, Hrdina, Montaser, & Hebeish, 2011) also concluded that the swelling of these hydrogels, synthesized by freeze-thawing for

the release of a model antibiotic, sparfloxacin, is pH dependent. In the meanwhile, their hydrogels displayed a positive effect towards the inhibition of Gram-positive and Gram-negative bacterial growth. Indeed, the release of sparfloxacin also relies on both pH and temperature. Yang et al. (J. M. Yang, Su, & Yang, 2004) prepared PVA/CH hydrogel membranes in various ratios and treated them with formaldehyde as crosslinking agent: their results showed that thermostability of the hydrogel membranes was enhanced, while PVA crystallinity decreased. Moreover, it was demonstrated that CH content variation and treatment with formaldehyde has no influence in the antibacterial assessment. Yang et al (X. Yang, Liu, Chen, Yu, & Zhu, 2008) also reported about the use of PVA/CH hydrogels for wound dressing, obtained by γ-irradiation combined with freeze-thawing. They found that hydrogels obtained by irradiation plus freeze-thawing show larger swelling capacity and mechanical strength, higher thermal stability, lower water evaporation rate, and are less turbid than those made by pure freeze-thawing and freeze-thawing followed by irradiation. The same hydrogels presented also good antibacterial activity against *Escherichia coli*. Zu et al. (Zu et al., 2012) synthesized PVA/CH hydrogels by using glutaraldehyde as crosslinking agent, with the aim of testing their use for transdermal drug delivery of nano-insulin. The hydrogel showed good mechanical and thermal properties, along with a high permeation rate of nano-insulin (4.421 ug/(cm^2 h)), suggesting high potential use of these hydrogels for non-invasive transdermal drug delivery system in diabetes chemotherapy.

Lignin is the second most abundant natural polymer next to cellulose, which makes up 20-30% of the cell walls of plants. It has always been utilized in low-value fields, for example heat and electricity generation. Nowadays, more researchers realise that its abundance could potentially resolve some environmental problems if we could successfully translate it into higher value material, including its use as antioxidant, UV absorbent, antimicrobial agent, reinforcement agent, carbon precursor and biomaterial for tissue engineering and gene therapy (Kai et al., 2016; Thakur, Thakur, Raghavan, & Kessler, 2014). A recent study reported the use of nanoparticles combining chitosan and lignosulfonates for cosmetic and biomedical uses (S. Kim et al., 2013). A greater stability to lysozyme degradation, antimicrobial activity and biocompatibility with human cells were concluded upon lignosulfonates incorporation into chitosan nanoparticles. No examples can be found where the antimicrobial activity of chi-

tosan/PVA based blend was tested in presence of lignin, which has been demonstrated to have inherent antimicrobial and antioxidant capabilities at the nanoscale. In this study, we synthesized PVA/CH hydrogels through a simple freeze-thawing approach and we aimed to study how different amount of lignin nanoparticles (LNP), incorporated in PVA/CH hydrogels, could modify not only blend microstructure, but also swelling, thermal, mechanical, antibacterial and antioxidant behaviour of reference blend.

2. Experimental section

2.1 Materials

Polyvinyl alcohol (PVA) (Mw 124–146 kg mol^{-1}, 99% hydrolyzed), a synthetic and biodegradable polymer produced by the hydrolysis of polyvinyl acetate, was supplied by Sigma–Aldrich® (Italy). High molecular weight chitosan (CH), (practical grade, Batch MKBB0585, degree of deacetylation >77%, viscosity: 1220 cPs, 1.61×10^5 Da), was supplied by Sigma–Aldrich® (Italy).

Pristine lignin, obtained as bio-residue of conversion of *Arundo donax L.* biomass to bioethanol in a steam explosion pre-treatment, followed by enzymatic reactions and filtration, was supplied by CRB (Centro Ricerca Biomasse, University of Perugia) (Cotana et al., 2014).

Lignin nanoparticle (LNP) suspension was prepared from pristine lignin by hydrochloric acidolysis, as presented in our previous work (Weijun Yang, Kenny, & Puglia, 2015). All the chemical reagents were supplied by Sigma–Aldrich® and used as received.

2.2 Methods

2.2.1 Preparation of binary PVA/CH and ternary PVA/CH/LNP nanocomposite hydrogels

PVA/CH (90/10) hydrogel was prepared according to the following steps: PVA was diluted in deionized water at 20% (wt/v) under magnetic stirring at 90 °C for 4 h, while chitosan was dissolved in water with glacial acetic acid (1% v/v) under magnetic stirring at 40 °C for 12 h. According to the designed blending ratio, the mixed solutions of chitosan and PVA were sonicated (Vibracell 75043, 750W, Bioblock Scientific) for 5 min at 40% of amplitude and homogeneous solutions were obtained and poured into a Petri dish cover by a Teflon® sheet.

After that, the poured material was kept at -30°C for 18 hours to freeze and then exposed at 25 °C to thaw for 4 hours to complete one cycle. The hydrogels were prepared by repeating four times the above freezing/thawing cycles and, with the freezing step exceeded 18 h in the final cycle. In order to eliminate the free polymer molecules not incorporated into the cross-link regions and the free ions, all the hydrogels were soaked in pure water for several times. PVA/CH/LNP ternary nanocomposite hydrogels containing 10 wt % of chitosan and 1 and 3 wt % of LNP were also prepared, by using the following protocol: PVA was diluted in deionized water at 20% (wt/v) under magnetic stirring at 90 °C for 4 h and a specific amount of the aqueous dispersion of LNP was added in order to obtain the selected percentage of LNP respect to the PVA matrix. The mixture was then sonicated (Vibracell 75043, 750W, Bioblock Scientific) for 5 min at 40 % of amplitude. Chitosan was separately dissolved in water with glacial acetic acid (1% v/v) under magnetic stirring at 40 °C for 12 h and then added to the PVA/LNP solution previously obtained. The final mixture was again sonicated for 5 min at 40% of amplitude and cast into a Petri dish cover by a Teflon® sheet. Five consecutive freeze (-30 °C, 18 hour) and thaw (room temperature, 4 h) cycles, with a longer final freeze cycle, were also considered in this case.

2.2.2 Morphological analysis

The microstructure of hydrogel cross-sections was observed by using a Field Emission Scanning Electron Microscope, FESEM, Supra 25-Zeiss. Specimens were cryo-fractured by immersion in liquid nitrogen and mounted on copper stubs perpendicularly to their surface. Samples were gold coated and observed by using an accelerating voltage of 4 kV.

2.2.3 Differential scanning calorimetry (DSC)

Hydrogel samples of about 3 mg were tested by using a differential scanning calorimeter (DSC, TA Instrument, Q200). Measurements were carried out under nitrogen flow in the temperature range from -25 °C to 220 °C at a heating rate of 10 °C/min. After a first heating step, cooling and second heating were performed. Data were recorded both during the cooling and second heating steps. The glass transition temperature (T_g) was taken as the inflection point of the specific heat increment at the glass-rubber transition, while the melting tempera-

ture (T_m) and melting enthalpy (ΔH_m) were determined during the cooling and the 2nd heating scan, respectively. Three samples were used to characterize each material.

2.2.4 Thermogravimetric analysis (TGA)

TGA tests were carried out using a thermo gravimetric analyzer (TGA, Seiko Exstar 6300). The samples, approximately 3 mg, were heated from 30 to 600 °C at a heating rate of 10°C/min under nitrogen atmosphere. The peak values temperatures and 20% of weight loss temperature ($T_{20\%}$) were obtained from derivative thermogravimetric (DTG) data, along with the residual weight at 600 °C.

2.2.5 Dynamic mechanical thermal analysis (DMTA)

Dynamic mechanical properties of hydrogel samples were measured using ARES N2 instrument (Reometric Scientific) in torsion mode on a plate with a diameter of 8 mm. The storage (G'), loss modulus (G'') and viscosity (Log η) values were obtained at a constant temperature of 25 °C, strain amplitude of 1.0% and over a frequency range from 0.1 to 100 Hz.

2.2.6 Swelling studies

The swelling behavior of the nanocomposite hydrogels was measured in water (pH = 7.0) at 25 °C. The swelling ratio (SR, %) for each sample was calculated by applying the Eq. (1):

$$SR = \frac{M_S - M_D}{M_S} \times 100 \qquad (1)$$

where M_S and M_D are the hydrogel masses in the swollen and in the dry state, respectively. All measurements were repeated four times.

2.2.7 Antiradical activity of migrating substances

DPPH radical scavenging activity for PVA/CH and ternary PVA/CH/LNP hydrogels containing different amount of lignin nanoparticles was determined according to the method proposed by Byun et al. (Chernoberezhskii, Atanesyan, Dyagileva, Lorentsson, & Leshchenko, 2002) and Domenek et al. (Domenek, Louaifi, Guinault, & Baumberger, 2013) with a slight modification. Hydrogel (0.1 g) was cut into small pieces and immersed in 2 mL of methanol

for 24 h at ambient temperature. The supernatant obtained was analyzed for evaluation of DPPH radical scavenging activity: methanol extract (1 mL) was mixed with 1 mL of DPPH in methanol (50 mg L^{-1}), resulting in a 25 mg L^{-1} DPPH concentration solution. The mixture was maintained at room temperature in the dark for 60 min. The absorbance was measured at 517 nm using a UV-Vis spectrometer (Varian, Cary 4000). The mixture solution of methanol extracted from neat PVA and DPPH methanol was used as control. DPPH radical scavenging activity was calculated by using Equation 2, where A_{sample} was the absorbance of sample and $A_{control}$ was the absorbance of the control.

$$(RSA,\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] * 100 \qquad (2)$$

2.2.8 Antimicrobial tests: bacterial strain culture conditions and viability assays.

Escherichia coli RB *(E. coli* RB), *Staphylococcus aureus* 8325-4 *(S. aureus* 8325-4) and *Staphylococcus epidermidis* RP62A *(S. epidermidis* RP62A) were used in this study. The former was used as main representative of Gram negative bacteria, the latter two instead as representatives of Gram positive bacteria. *E. coli* RB was kindly provided by the "Istituto Zooprofilattico di Pavia" (Italy) whereas *S. aureus* 8325-4 and *S. epidermidis* RP62A were kindly supplied by Timothy J. Foster (Department of Microbiology, Dublin, Ireland). *E. coli* RB was routinely grown in Luria Bertani Broth (LB) (Difco, Detroit, MI, USA), *S. aureus* 8325-4 in Brian Heart Infusion (BHI) (Difco) and *S. epidermidis* RP62A in Tryptic Soy Broth (TSB) (Difco) overnight under aerobic conditions at 37 °C, 200 rpm (Certomat® BS-T, B.Braun Biotech International). To evaluate the antimicrobial activity of the generated hydrogels in planktonic conditions, the overnight cultures were diluted in fresh appropriate medium and 150µL of diluted bacterial suspension were deposited on sterilized hydrogels placed at the bottom of a 96-well flat-bottom polystyrene tissue culture plates (TCPs) well. 5 x 10^4 bacteria/150 µL suspensions, obtained by comparing the O_{D600} of the overnight culture with a standard curve correlating O_{D600} to cell number, were used to test the antibacterial activity at 37 °C for 24 hours. The samples were then incubated at 37 °C for 24 hours in static conditions. Similarly, TCP empty wells were incubated with the same bacterial suspension at the same temperature and time and used as positive control. At the end of the culturing period, the bac-

terial viability was assayed through the quantitative 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazoliumbromide (MTT) (Sigma Aldrich®, StLouis, MO, USA) test, as previously reported (Yalcinkaya et al., 2017). Briefly, this colorimetric assay measures dehydrogenase activity, as an indicator of the metabolic state of the cells. After the indicated culturing times, bacterial suspensions were transferred to a new plate and cell viability assessed. 5 mg/mL of MTT solution, dissolved in PBS (0.134 M NaCl, 20mM Na_2HPO_4, 20 mM NaH_2PO_4), was used as stock solution and the working concentration was 0.5mg/mL. Bacteria were incubated in the presence of MTT solution at 37 °C for 3 hours. Upon presence of viable cells, reduction of MTT salt results in purple insoluble formazan granules. These precipitates were dissolved through acidified 2-propanol (0.04 N HCl). The result was recorded through an iMark® Microplate Absorbance Reader (Bio-Rad) at 562 nm with the reference wavelength set at 655 nm. Bacterial survival was expressed as percentage of the number of bacteria survived on the generated hydrogels to number of bacteria grown on TCP wells. Experiments were performed twice with triplicate samples.

2.2.9 Scanning electron microscopy (SEM) analysis.

Images of *E. coli* RB, *S. aureus* 8325-4 and *S. epidermidis* RP62A grown on LNP-enriched PVA/CH hydrogels were prepared essentially as already reported (Yalcinkaya et al., 2017). Briefly, all strains used in this study were incubated on previously sterilized hydrogels for 24 hours at 37°C. As a control, a Thermanox™ coverslip (Nunc™) was used to deposit both bacterial cells. Following incubation, samples were washed carefully with sterile water and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 1 h at + 4 °C. After additional washing with cacodylate buffer, the samples were dehydrated using increasing concentrations of ethanol (25, 50, 75%) for 5 min and final two washes of 10 minutes in 96% ethanol. The samples were then lyophilized for 6 hours using an Emitech K-850 apparatus, placed on a mounting base and sputter coated with gold (300 nm). Analysis was performed using a Zeiss EVO-MA10 scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

2.2.10 Biofilm formation and viability analysis

Since *S. epidermidis RP62A* is a biofilm producer strain, we explored whether the generated hydrogels were able to inhibit biofilm formation. To induce biofilm formation, overnight grown bacteria were diluted 1:200 in TSB-medium containing 0.25% glucose as previously described (Pallavicini et al., 2014; Saino et al., 2010; Sbarra et al., 2009; Taglietti et al., 2014) . An aliquot 150µl of diluted bacterial suspension was deposited on each hydrogel and incubated for 24 hours at 37°C. As positive control, TCPS wells were incubated with the same amount of diluted bacterial suspension at the same incubation conditions. The viability of *S. epidermidis* was evaluated as described above through MTT test after mechanical disruption of the biofilm. The results of the TCPS wells (control) were set to 100%. The experiments were performed twice with triplicate samples.

3. Results and discussion

3.1 Microstructure of produced hydrogels

The morphologies of the freeze-dried hydrogels containing different amount of LNP are shown in **Figure 1a**.

The PVA/CH hydrogel has an interconnecting porous structure, with macrovoids whose walls are rough, whereas a porous honeycomb-like structure is shown for the hydrogels containing LNP. It was supposed that the well dispersed lignin nanoparticles could serve as nucleation agents (Lee et al., 2005), in the meanwhile, the hydrophobic nature of lignin and strong interaction between PVA/CH molecules and LNP prevents the PVA molecules from moving and dissolving into the water, resulting in promoting the crosslinking effect (Xia, Yih, D'Souza, & Hu, 2003). Consequently, more uniform and higher quantity of pore microstructures formed. The pore diameter of PVA/CH/1LNP and PVA/CH/3LNP hydrogels is about 10-20 μm, which indicates a good accessibility of water into the amorphous regions of the hydrogels. This result demonstrates that LNP had a role in the formation of pores for the PVA/CH hydrogel. To further magnify the studied hydrogels, some LNP aggregates (arrows) could be seen on the pore-wall surface in PVA/CH/1LNP and PVA/CH/3LNP hydrogels, more visible in PVA/CH/3LNP system (**Figure 1b**).

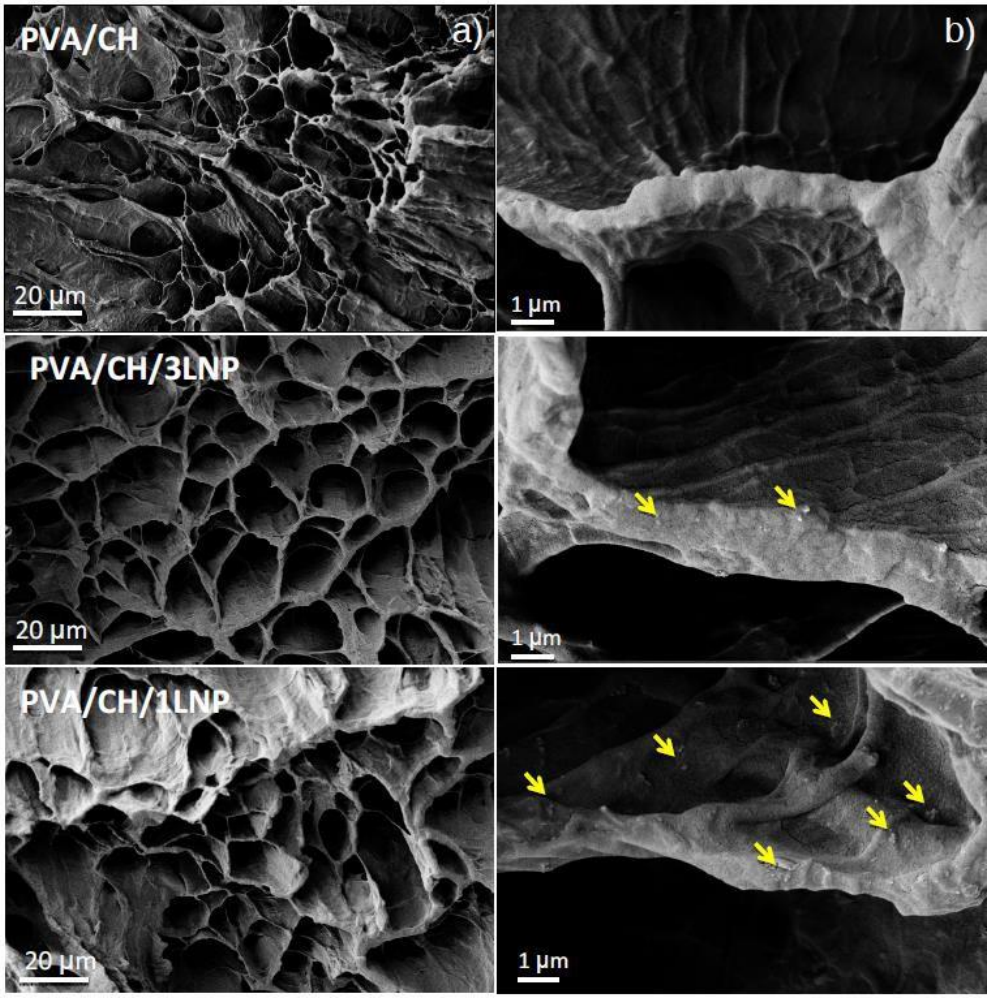

Figure 1: Morphologies of freeze-dried hydrogels containing different amount of LNP (a) and magnification of LNP aggregates (b).

3.2 Differential scanning calorimetry

DSC analysis was conducted to analyze the thermal properties of PVA/CH binary and ternary nanocomposite hydrogels containing LNP. The values of thermal parameters obtained in the cooling and second heating scans for all samples are summarized in **Table 1**, while DSC cooling and 2nd heating curves are reported in **Figure 2(a-b)**. A single composition-dependent glass transition temperature (T_g) is indicative of blend miscibility, even in presence of lignin nanoparticles. The addition of the LNP did not change the crystallization temperature of PVA/CH binary hydrogel, but lead to ΔH_m increase from 42.8 (PVA/CH) to 53.6 J/g (PVA/CH/1LNP), indicating that higher crystalline region was achieved and enhanced thermal stability has been achieved by adding LNP. PVA is a semicrystalline polymer bearing plenty of hydroxyl groups which form inter- and intra-molecular hydrogen bonds. Since LNP also have a lot of hydroxyl groups in their structures, strong molecular interactions (hydrogen

bonding or dipole-dipole interactions) between polymer and LNP can be expected (Hu, Ye, Tang, Zhang, & Zhang, 2016; Kubo & Kadla, 2003; Ye, Jiang, Hu, Zhang, & Zhang, 2016). However, addition of 3 wt % did not further increase the ΔH_m, the result could be understood when taking into account the abundant LNP agglomerates detected by SEM.

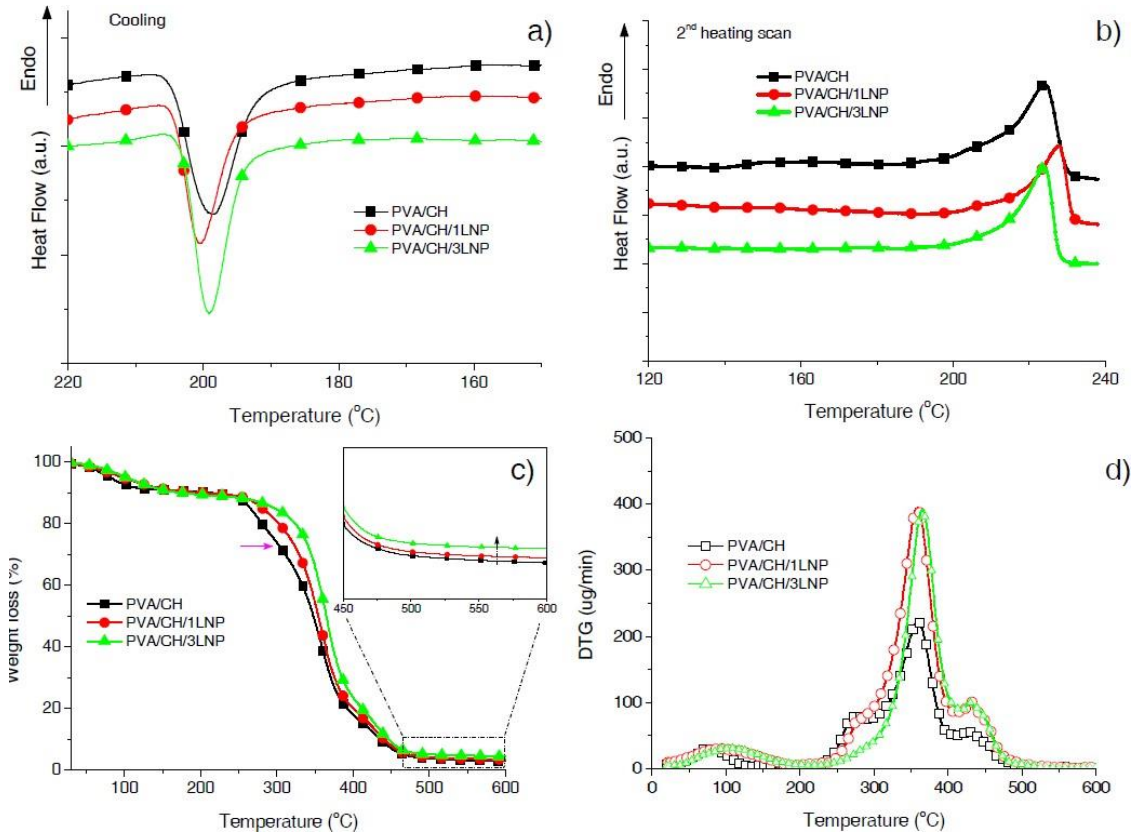

Figure 2: DSC cooling (a) and 2nd heating curves (b) for PVA/CH hydrogels containing different amount of LNP; TG (c) and DTG curves (d) of pure PVA binary and ternary system hydrogels with different LNP loading.

Table 1: DSC and TGA thermal parameters of PVA/CH blend and PVA/CH/LNP hydrogel ternary systems

	T_g (°C)	T_g (°C)	T_m (°C)	ΔH_m (J/g)	Peak 1 (°C)	Peak 2 (°C)	Peak 3 (°C)	$T_{20\%}$ (°C)	Residue at 600°C (%)
PVA/CH	-	-	225.0±1.1	42.8±0.5	77.3	273.8	359.6	280.5	2.9
PVA/CH/1LNP	73.6±2.0	73.6±2.0	226.9±1.2	53.6±0.0	89.6	275.4	359.2	302.5	3.5
PVA/CH/3LNP	74.5±2.1	74.5±2.1	224.4±1.0	48.0±1.0	106.5	-	365.5	324.1	4.5

3.3 Thermogravimetric analysis

TG and DTG curves of pure PVA binary and ternary hydrogels were recorded in order to in-

vestigate the effect of LNP loading on the thermal degradation behaviour of resulted composites (**Figure 2c-d**). The peak values of DTG and the residual weight at 600 °C, were also summarized in **Table 1**. The degradation progress was divided into four steps: 1) the small weight loss (peak 1) at 75-100 °C (about 10%) is due to the loss of adsorbed, bound water and residual acetic acid; 2) The weight loss (peak 2) at 200-300 °C was attributed to the initial thermal decomposition of CH and PVA; 3) the maximum decomposition range between 300 and 400 °C (peak 3) could be attributed to a complex degradation process of PVA and CH, including the dehydration of the saccharides rings and the depolymerization of the acetylated and deacetylated units of the polymer (J. M. Yang, Su, Leu, & Yang, 2004); 4) weight loss at 400-450 °C (peak 4) is related to the thermal degradation event of some by-products generated by PVA (Bonilla, Fortunati, Atarés, Chiralt, & Kenny, 2014; Chen, Wang, Mao, Liao, & Hsieh, 2008). Evidently, with increasing dose of LNP from 0 to 3 wt %, the peak 1 shifted from 77.3 up to 106.5 °C: this effect can be attributed to the pore-forming effect of LNP in PVA/CH hydrogel, which efficiently delayed the removal of water and residual acetic acid. Similar results were observed in our previous study with wheat gluten in presence of glycerol (Weijun Yang et al., 2015). Interestingly, the peak 2 became less visible when 1 wt % of LNP was used, and disappeared when 3 wt% of LNP was added, confirming the delayed decomposition process of PVA and CH due to the addition of LNP. Furthermore, the $T_{20\%}$ also dramatically rose from 280.5 to 324.1 °C, along with increase in the weight residue at 600 °C from 2.9 to 4.5 %.

3.4 Dynamic mechanical thermal analysis

Figure 3a-b and **Table 2** show the storage modulus (G'), loss modulus (G'') and viscosity (Log η) values versus scan frequency for various hydrogel samples. The PVA/CH shows the lowest G', G'' and viscosity values in all the frequency range. After the addition of 1 wt % of LNP, the G', G'' and viscosity increased from 34.8 KPa, 1.6 KPa and 34.9 KPa·s to 63.4 KPa, 4.1 KPa and 63.5 KPa·s at 1 Hz frequency, increasing by 82.2, 156.3 and 81.9 %, respectively. The viscosity of the studied hydrogels decreased linearly with the increase of the frequency, which was in consistence with previous results (Tang, Du, Hu, Shi, & Kennedy, 2007).

Table 2: G' and G'' values for PVA/CH blend and PVA/CH/LNP hydrogel ternary systems at two different frequencies

Materials	*G'*(KPa)		*G''* (KPa)		η (KPa · s)	
Frequency (Rad/s)	1	10	1	10	1	10
PVA/CH	34.8±0.8	36.3±0.5	1.6±0.5	1.9±0.5	34.9±5.7	3.6±0.1
PVA/CH/1LNP	63.4±8.0	64.3±8.0	4.1±0.8	4.8±1.0	63.5±10.0	6.4±1.5
PVA/CH/3LNP	62.2±3.7	64.8±3.4	3.1±0.7	3.5±0.7	62.3±3.6	6.5±0.3

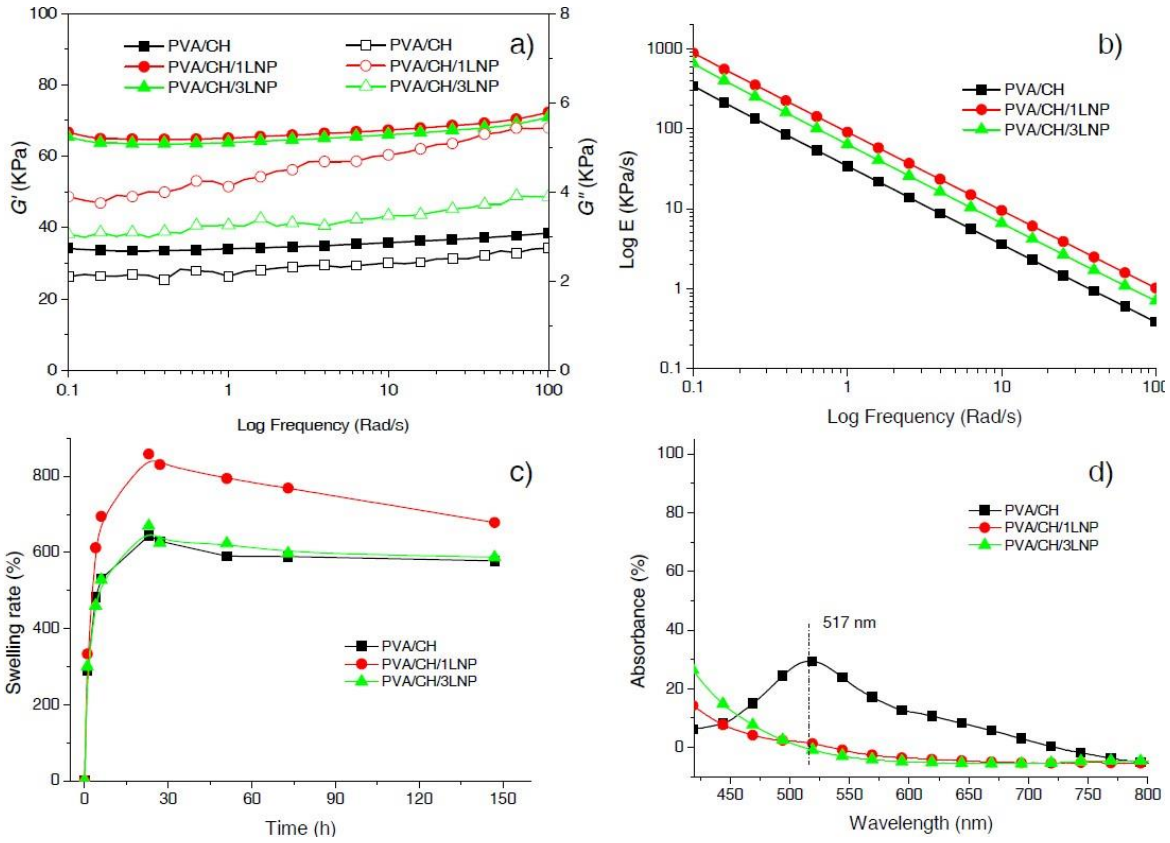

Figure 3: Curves for Storage modulus (G'), loss modulus (G'') (a) and viscosity (Log η) (b) vs. frequency for PVA/CH hydrogels containing different amount of LNP; swelling behaviour of pure PVA binary and ternary system hydrogels with different LNP loading (c) and antioxidant response of migrating substances for different nanocomposite hydrogels directly immersed into the methanol solution for 24 h (d).

This result demonstrates that LNP could efficiently enhance the mechanical properties of PVA/CH hydrogel, which may due to the strong interactions between PVA/CH and LNP. Although the PVA/CH and lignin are immiscible in the bulk, the results showed the existence

of some specific intermolecular interaction (like hydrogen bonding or dipole-dipole interactions) between them.

The same results were also confirmed by Korbag et al. (Korbag & Mohamed Saleh, 2016), that used nuclear magnetic resonance and FTIR spectroscopy techniques. Recently, Ye et al. (Ye et al., 2016) and Hu et al. (Hu et al., 2016) also found that PVA/lignosulfonate composites had preferable mechanical properties than pure PVA, due to the primary rigidity of nanolignin structures and the formation of strong hydrogen bonds. However, when the loading of LNP rose up to 3 wt %, the G', G'' and Log η shows steady even slightly decline with respect to PVA/CH/1LNP hydrogel. This result could be responsible for the abundant formation of LNP aggregates on the surface of hydrogels (Figure 1b).

3.5 Swelling behavior

PVA/CH hydrogels have lots of hydrophilic groups, which show high accessibility to water, but these polymer networks can be avoided dissolving in water due to the formed chemical or physical bonds between polymer chains. Hydrogels absorb water and become fully swollen state, which is common to living tissues in some physical properties (Hamidi, Azadi, & Rafiei, 2008). Results related to the influence of LNP (presence and content) on swelling behavior of the PVA/CH hydrogels are presented in **Figure 3c**. The results show that the swelling behavior of physically crosslinked hydrogels is influenced by the addition of lignin nanoparticles. Compared with PVA/CH hydrogel, PVA/CH/1LNP exhibited higher and faster swelling rate, until reaching the maximum (856.6 %) after 23 hours (644.2 % for PVA/CH hydrogel). It is axiomatic, since more uniform micropores (Figure 1b) indicates higher affinity for water. However, when the loading of the LNP increased to 3 wt%, the swelling rate reduced, despite a relative more homogeneous pore structure formed. This consequence should be ascribed to the hydrophobic nature of the lignin, which has been confirmed in our previous works (Fortunati et al., 2016; Frigerio, 2014; Weijun Yang et al., 2015). After 23 h, the swelling rate decreased gradually, which was due to the migration and dissolving of some PVA into water.

Generally, the swelling property is highly related to the crosslinking degree between the PVA molecules, i.e., the swelling capability will decrease with the increase of the crosslinking degree. At the same time, higher crosslinking degree is beneficial to the mechanical prop-

erties of the hydrogels (Tong, Zheng, Lu, Zhang, & Cheng, 2007). In this study, it is supposed that the strong interaction between PVA/CH molecules and LNP prevents the PVA molecules from moving and dissolving into the water, resulting in promoting the crosslinking effect. Consequently, more uniform and higher quantity of pore microstructures formed, increasing the swelling property and reinforcing the mechanical behaviours. The schematic diagram has been presented in **Figure 4**. Some other researchers also obtained the common results by adding layered silicates (Xia et al., 2003) and carbon nanotube (Tong et al., 2007) into the hydrogels.

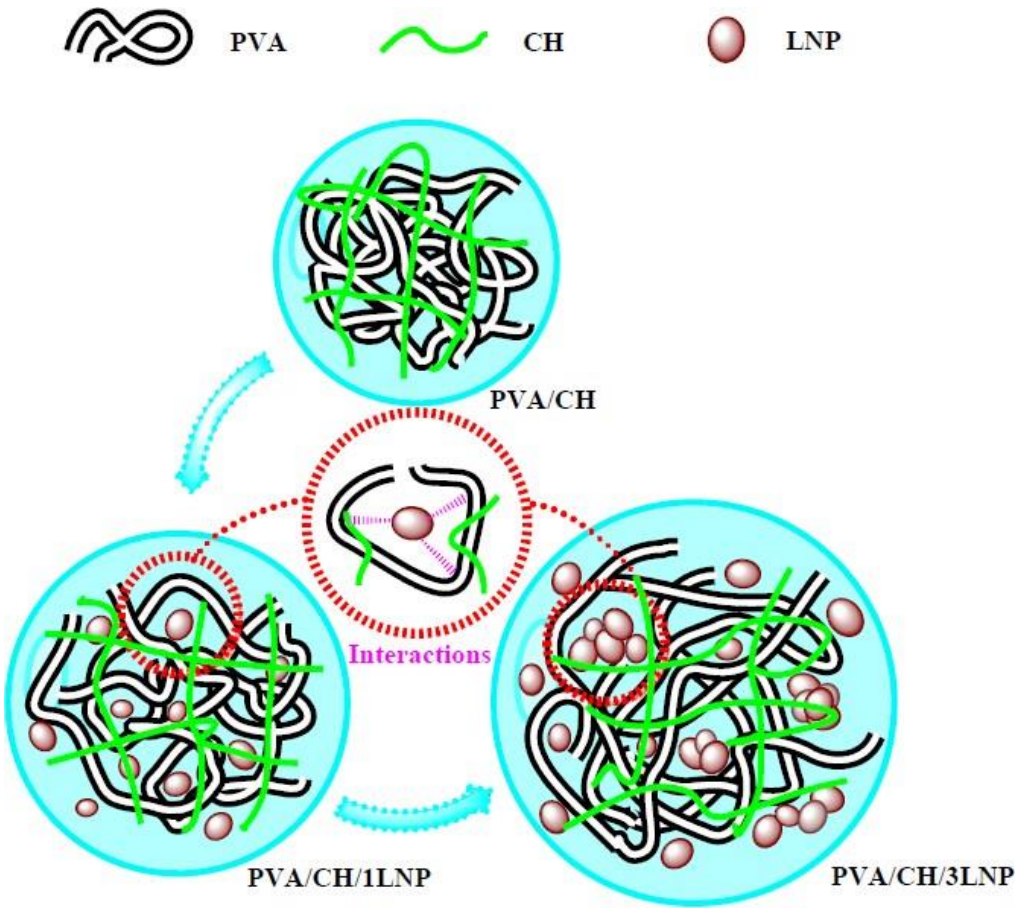

Figure 4: Schematic diagram showing the interactions between PVA/CH molecules and LNP nanoparticles

3.6 Antiradical activity of migrating substances

Figure 3d shows the antioxidant response of migrating substances for different nanocomposite hydrogels directly immersed into the methanol solution for 24 h. The pure PVA samples

were regarded as control samples and did not show any DPPH radical scavenging activity, as expected (not given in curve). When mixing with 10 wt% of chitosan, the absorption value at 517 nm of PVA/CH hydrogel decreased to 25.3 from 35.6%, with RSA value of 28.9%, highlighting a good antioxidation response. Antioxidant properties of chitosan derivatives have been studied (K. W. Kim & Thomas, 2007; Xie, Xu, & Liu, 2001). The scavenging mechanism of chitosan is based on the fact that the residual free amino (NH_2) groups can react with free radicals to form stable radicals, afterwards NH_2 groups form ammonium (NH_3^+) groups by absorbing the hydrogen ions from the solution (Yen, Yang, & Mau, 2008). With the addition of 1 wt % LNP (PVA/CH/1LNP hydrogel), a more remarkable DPPH scavenging activity could be obtained (74.3%, absorption 9.2%). In the hydrogel containing 3 wt % LNP (PVA/CH/3LNP), the antioxidant activity increased up to 78.6% (absorption 7.6%), and the mechanism for antioxidation effect of LNP has been well discussed in our previous study (W. Yang et al., 2016). Lignin nanoparticles can serve as antioxidation controlled release agents in PVA/CH hydrogel.

3.7 Antibacterial activity

Since it was previously reported that lignin extracts (Dong et al., 2011; S. Kim et al., 2013) and LNP (W. Yang et al., 2016) showed antibacterial activity on different bacterial strains, we decided to verify whether LNP affected bacterial growth also upon incorporation into hydrogels. Therefore, we selected two laboratory strains representing Gram positive and negative classes, i.e. *Staphylococcus aureus* 8325-4 and *Escherichia coli* RB, respectively. We analyzed the effect of the generated hydrogels on the growth of these two strains at 37°C for 24 hours, thus in the optimal condition for their growth. As shown in **Figure 5a** a drastic reduction in bacterial viability was observed for both bacterial strains. In the case of *E. coli* the growth is reduced more than 95% compared to the control upon incubation on all three types of nanocomposites ($p<0.001$). Regarding *S. aureus*, a reduction greater than 85% on PVA/CH blend is observed ($p<0.001$), whereas on PVA/CH/1LNP and PVA/CH/3LNP the decrease in bacterial survival is even more marked (more than 95%) ($p<0.001$). These data led us to speculate that already the neat hydrogel bearing CH without LNP may already display an antibacterial activity.

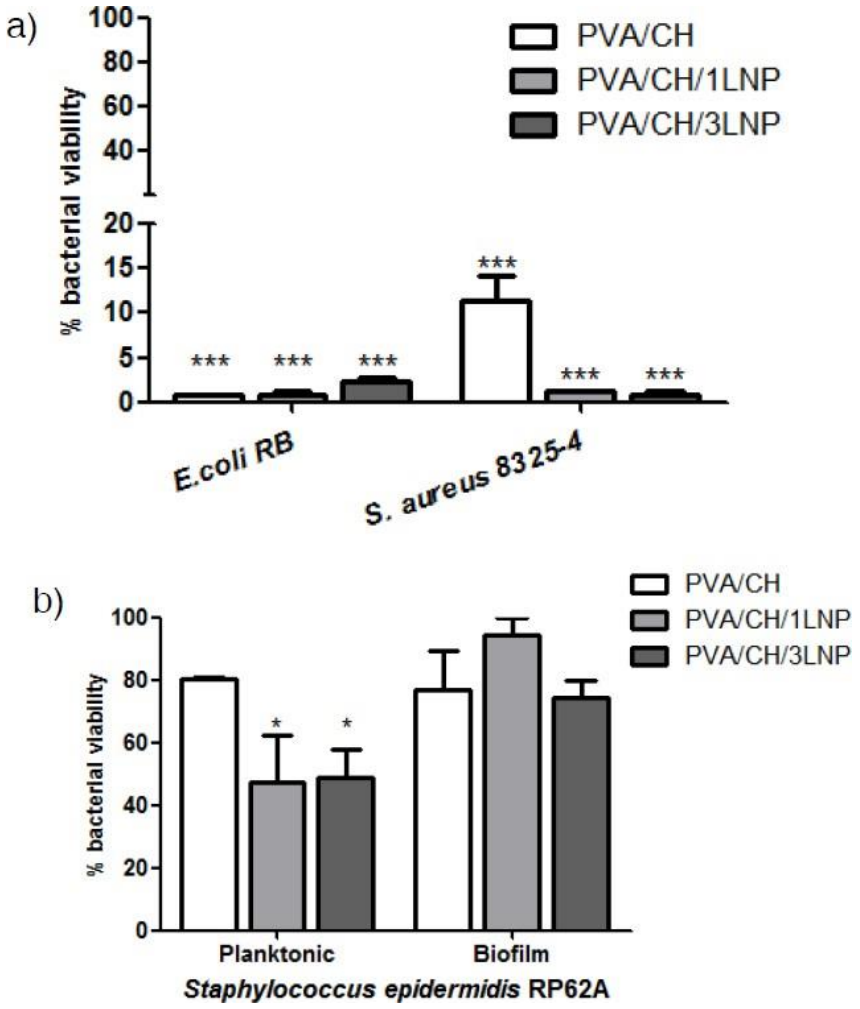

Figure 5: Bacterial viability of *E. coli* strain RB and *S. aureus* strain 8325-4 on different nanocomposite hydrogels at 37°C for 24 hours (a). Data are presented as viability percentage to TCPs reference set equal to 100%. One-way ANOVA with Bonferroni post-test was performed to evaluate statistical significance. All data were compared with TCP reference (***=$p<0.001$); Bacterial viability of *S. epidermidis* strain RP62A on different nanocomposite hydrogels at 37°C for 24 hours in both planktonic and biofilm-forming conditions (b). Data are presented as viability percentage to TCPs reference set equal to 100%. One-way ANOVA with Bonferroni post-test was performed to evaluate statistical significance. All data were compared with TCP reference (*=$p<0.05$)

As a matter of fact, CH has been shown to exert antibacterial activity in different formulations (Dong et al., 2011; S. Kim et al., 2013; Pelgrift & Friedman, 2013), even in the contest of hydrogels (Abdel-Mohsen et al., 2011). In this case, Abdel-Mohsen and colleagues [9] tested different PVA/CH ratios, showing that the lowest one for CH (80/20) was affecting only *E. coli* growth as tested by agar diffusion test. In contrast, our results are slightly different: even if the ratio we used in this study is even lower compared to the one tested by Abdel-Mohsen

et al (Abdel-Mohsen et al., 2011), our data suggest that CH may have an effect already alone and LNP nanofilling may have an addictive effect, especially on *S. aureus* cells.

However, it has to be underlined that we used an assay that measures the metabolic activity of live bacteria, thus being more sensitive than agar diffusion test. Furthermore, the antimicrobial activity of the generated hydrogels against *S. epidermidis* RP62A, a coagulase-negative *Staphylococcus* able to form a strong biofilm (Christensen, Baddour, & Simpson, 1987), was evaluated. Therefore, hydrogels affected *S. epidermidis* viability both in planktonic and in biofilm-forming conditions (**Figure 5b**). As shown, a significant ($p<0.05$) antibacterial action is observed only on LNP-enriched hydrogels in planktonic conditions, instead PVA/CH nanocomposite was not affecting *S. epidermidis* growth. Upon testing of hydrogels efficacy against biofilm formation, none of three blends displayed a significant antibiofilm activity. This reflects the much stronger well-known resistance of biofilm-embedded bacteria to exposure to any type of stress. Furthermore, it is noteworthy that the increased concentration of LNP in PVA/CH/3LNP blend reduced bacterial viability, but to level comparable to PVA/CH nanocomposite especially in biofilm-forming conditions for *S.epidermidis* strain. In our culture conditions, both CH and LNP seems to be ineffective against biofilm formation. It may be necessary to increase the content of both components in the nanocomposites in order to be more efficacious.Lastly, the adhesion to the produced hydrogels of all three used bacterial strains was investigated by SEM observation. As reported in **Figure 6,** both staphylococcal strains showed great adherence to the substrate, possibly owing to their many surface adhesion proteins (Foster, Geoghegan, Ganesh, & Hook, 2014). Even if SEM images seem to be in contrast with viability assay, we need to point out that microscopy observation does not inform about viability: bacterial cell are shown attached to the surface but it does not mean that those cells are alive. By contrast, *E.coli* RB showed extremely limited adhesion ability, being almost absent on all three hydrogels indirectly confirming and supporting the data on cell viability.

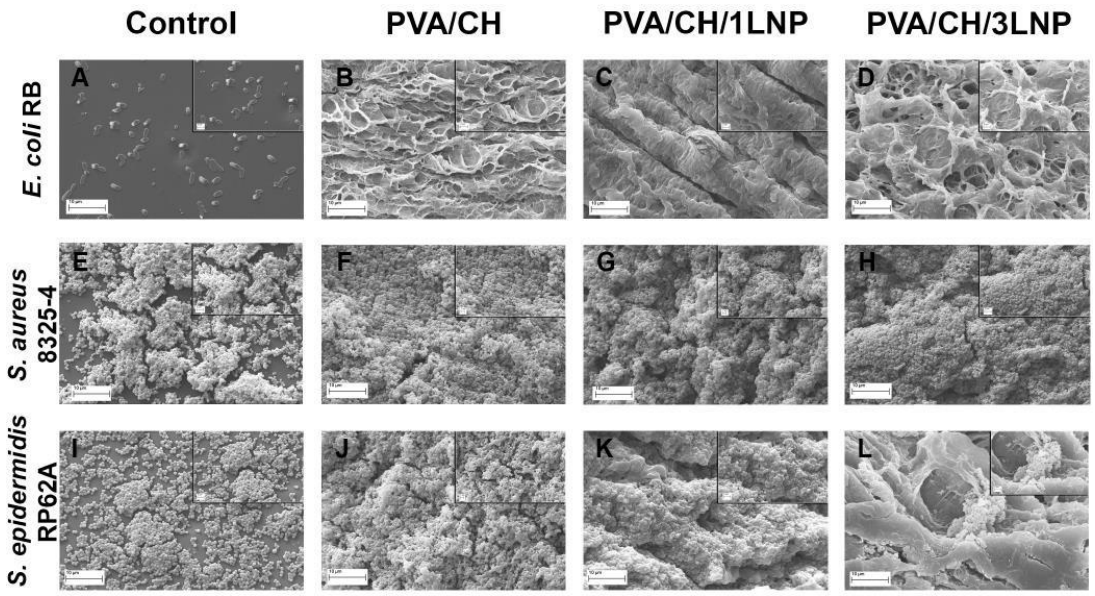

Figure 6: SEM representative images of *E. coli* strain RB (panels B, C and D), *S. aureus* strain 8325-4 (panels F, G and H) and *S. epidermidis* RP62A (panels J, K and L) cultured on the generated LNP-enriched PVA/CH hydrogels for 24 hours at 37°C. Plastic control is also shown for each bacterial strain (panels A, E, I).

4. Conclusions

PVA/CH hydrogels containing 1 and 3 wt% of LNP were successfully prepared by applying a freezing-thawing procedure. It was observed that more uniform and higher porosity (pores with diameter about 10-20 μm), formed in the presence of LNP, facilitated the accessibility of water into the amorphous regions of the hydrogels. The results demonstrated that 1 wt % of LNP could efficiently enhance both the thermal and mechanical properties of PVA/CH hydrogels, due to the strong interactions between PVA/CH and LNP obtained during the applied process, whereas the presence of some agglomerates limited the effect in the case of 3 wt% LNP based formulations. The swelling studies underlined that the strong obtained interaction between PVA/CH molecules and LNP prevents the PVA molecules from moving and dissolving into the water, resulting in promoting the crosslinking effect. Moreover, LNP revealed to be efficient in terms of antioxidative response, serving as release agents in synergism with CH in the PVA/CH. Chitosan and LNP components showed to be also both effective against *E.coli* and *S.aureus* bacteria strain, suggesting the possible use of PVA/CH hydrogels incorporating LNP in biomedical and packaging sectors.

Acknowledgements

W. Yang appreciates the funding support from China Scholarship Council (CSC). The authors also appreciate the Scanning Electron Microscopy (SEM) characterization from Sapienza University of Rome. This research was performed within the framework of TD COST Action TD1305 "Improved Protection of Medical Devices Against Infection (IPROMEDAI)" (http://www.cost.eu/COST_Actions/tdp/TD1305).

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