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PVA bio-nanocomposites: A new take-off using cellulose nanocrystals and PLGA nanoparticles

N. Rescignano[a], E. Fortunati[b,*], S. Montesano[c], C. Emiliani[c], J.M. Kenny[a,b], S. Martino[c], I. Armentano[b]

[a] Institute of Polymer Science and Technology, ICTP – CSIC, Madrid, Spain
[b] Materials Engineering Center, UdR INSTM, Strada di Pentima 4, 05100 Terni, Italy
[c] Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy

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ABSTRACT

The formation of a new generation of hybrid bio-nanocomposites is reported: these are intended at modulating the mechanical, thermal and biocompatibility properties of the poly(vinyl alcohol) (PVA) by the combination of cellulose nanocrystals (CNC) and poly (D,L-lactide-co-glycolide) (PLGA) nanoparticles (NPs) loaded with bovine serum albumin fluorescein isothiocynate conjugate (FITC-BSA). CNC were synthesized from microcrystalline cellulose by hydrolysis, while PLGA nanoparticles were produced by a double emulsion with subsequent solvent evaporation. Firstly, binary bio-nanocomposites with different CNC amounts were developed in order to select the right content of CNC. Next, ternary PVA/CNC/NPs bio-nanocomposites were developed. The addition of CNC increased the elongation properties without compromising the other mechanical responses. Thermal analysis underlined the nucleation effect of the synergic presence of cellulose and nanoparticles. Remarkably, bio-nanocomposite films are suitable to vehiculate biopolymeric nanoparticles to adult bone marrow mesenchymal stem cells successfully, thus representing a new tool for drug delivery strategies.

1. Introduction

There is a growing interest in drug delivery systems that can provide site-specific and continuous therapeutic drug levels for extended periods of time. These systems can been visioned as adhesive patches or implantable devices (Rescignano et al., 2013).

Poly(vinyl alcohol) (PVA) is the largest synthetic water-soluble polymer produced in the world (Ding et al., 2002). It is a hydrophilic and biocompatible synthetic polymer and it has been widely used in different areas of the biotechnological and biomedical fields due to its excellent chemical and physical properties, easy processing technique and low cytotoxicity (Chiellini, Corti, D'Antone, & Solaro, 2003). PVA is a highly versatile polymer offering a wide spectrum of property profiles opening the way of using it in a broad field of applications, among which is their use as a matrix for biodegradable composites. The nanocomposite approach has emerged in the last two decades as an efficient strategy to upgrade the structural and functional properties of synthetic polymers.

Generally, polymer nanocomposites are the result of the combination of polymers and inorganic/organic fillers at the nanometer scale (Armentano et al., 2013; Armentano, Dottori, Fortunati, Mattioli, & Kenny, 2010). A large variety of nanocomposites have been prepared using PVA as a matrix and nano-reinforcement such as layered silicate, silica, cadmium sulfide nanoparticles and carbon nanotubes (Guo, Ma, Hu, & Jiang, 2007; Paiva et al., 2004; Strawhecker & Manias, 2000; Wang, Ding, & Cheng, 2007). Normally, nanofillers used to prepare nanocomposites are inorganic and their processability, biocompatibility and biodegradability are much more limited than it is the case with those of organic nature (Armentano et al., 2010, 2013). Moreover, the use of cellulose nanocrystals (CNC) is increasing as the load-bearing constituent in developing new and inexpensive biodegradable materials due to their high aspect ratio. CNC have many additional advantages including a positive ecological effect, low density, low-energy consumption in manufacturing, ease for recycling by combustion, high sound attenuation and comparatively easy processability due to their non abrasive nature, which allows high filling levels, in turn resulting in significant cost savings. Moreover, CNC can be extracted from a wide variety of natural sources available throughout the world. The announcement of using cellulose nanocrystals from tunicin cellulose (structures from animal sources) as a reinforcing phase in a matrix, was firstly reported by Favier, Chanzy, and Cavaille (1995); the use of CNC from various sources such as ramie (Habibi & Dufresne, 2008), potato and starch (Dufresne, Cavaillé, & Helbert, 1997; Dufresne, Dupeyre, & Vignon, 2000), cotton and wood for the preparation of high performance composites has been

* Corresponding author. Tel.: +39 0744 492921; fax: +39 0744 492950.
E-mail address: elena.fortunati@unipg.it (E. Fortunati).

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extensively investigated (Brinchi, Cotana, Fortunati, & Kenny, 2013; Dufresne et al., 1997, 2000; Habibi & Dufresne, 2008; Okano, Bae, Jacobs, & Kim, 1990). To overcome the limited biological performance and to enhance the mechanical properties of PVA, a new class of engineering designed PVA bio-nanocomposites has been recently introduced, by incorporating cellulose nanocrystals (Ago, Okajima, Jakes, Park, & Rojas, 2012; Peresin, Habibi, Zoppe, Pawlak, & Rojas, 2010).

The hydrophilic nature of the polymer results in excellent interfacial compatibility between nanocrystals and polymer matrix, resulting in enhanced mechanical properties. The addition of cellulose nanostructures was found to produce 100% improvement in the tensile modulus of certain PVA nanocomposites (Qua, Hornsby, Sharma, Lyons, & McCall, 2009). However, transferring the exceptional mechanical properties of cellulose nanocrystals to the macro-scale properties of the bulk polymer still remains a challenge. Moreover, PVA has been also used as a hydrogel for application in tissue engineering and regenerative medicine fields, as matrices for tissue repairing and regenerating and for drug delivery. The release rates can be controlled through a composite drug release platform, which consists of nanoparticles containing drugs embedded within PVA films (Rosa, Iommelli, La Rotonda, Miro, & Quaglia, 2000). The implementation of nanotechnology offers new concepts for the development of optimized therapeutic and diagnostic tools in medicine (Verónica Lassalle, 2007). Biodegradable polymer nanoparticles (NPs) hold great promise to improve controlled and targeted drug delivery to the desired site of action. The close affinity of PVA with human system has made it a material of choice for different biomedical applications, such as the controlled release of drugs (Qua et al., 2009; Takamura, Ishii, & Hidaka, 1992) growth factors (Bourke et al., 2003), and proteins. However, the drug release from PVA films needs other controlled-release delivery systems (e.g., microspheres, nanoparticles, liposomes) that can be entrapped within these PVA systems without any detrimental effects that could occur in the presence of organic solvents and chemical cross-linking agents. The possibility to incorporate polymeric micro- and nano-spheres and other particulate delivery systems has numerous advantages, such as the isolation of the drug, slower drug release rates and the achievement of different drug release profiles, as well as the incorporation of multiple drugs in different microsphere populations.

Poly(D,L-lactide-co-glycolide) (PLGA), a food and drug administration (FDA) approved biodegradable polymer, has been widely investigated for drug delivery applications due to a number of advantageous features (Woo, Jiang, Jo, & DeLuca, 2001). First, its degradation rate can be tailored to obtain controlled delivery of drugs. Secondly, the material properties can be adjusted by changing the lactic acid/glycolic acid ratio or the molecular weight. Thirdly, PLGA nanoparticles can be formulated in order to load not only small molecules but also proteins and larger payloads (Chan et al., 2009; Jensen et al., 2010; Tang et al., 2009).

Our approach to extend the use of biodegradable polymer NPs as drug delivery systems is based on their encapsulation into PVA/cellulose nanocrystal bio-nanocomposites (PVA/CNC), thus offering a combination of two nanotechnologies. We designed, developed and characterized novel multifunctional bio-nanocomposites that combine the biocompatibility and the high mechanical response of the PVA/CNC systems with the protein control release exerted by polymeric PLGA nanoparticles. We used bone-marrow mesenchymal stem cells to evaluate the biocompatibility of both binary and ternary PVA nanocomposite films and to explore the release of NPs by those films. Our results demonstrated that stem cells up-take easily NPs released either in the culture medium by the binary films following the rapid PVA dissolution, or by ternary films through direct contact modality (stem cellular membranes-films).

2. Experimental

2.1. Materials

Polyvinyl alcohol (PVA) (31.000–50.000 g mol^{-1}, 87–89% hydrolyzed) was used as matrix for the nanocomposite preparation and the same polymer was used as surfactant in the polymeric nanoparticle synthesis from Sigma Aldrich. Poly(D,L-lactide-co-glycolide), PLGA (I.V. 0.95–1.20 dL g^{-1}) ether terminated, with a 50/50 ratio (PLA/PGA) supplied by Absorbables Polymers/Lactel (Durect Corporation), was used as biodegradable polymer for the nanoparticle preparation.

Microcrystalline cellulose (MCC, dimensions of 10–15 μm), supplied by Sigma Aldrich, was used as start material for the cellulose nanocrystal (CNC) synthesis.

The fluorescein isothiocyanate labelled bovine serum albumin (BSA-FITC) was purchased from Sigma Aldrich and used as a protein model. Chloroform ($CHCl_3$), from Sigma Aldrich, was used as solvent in the polymeric nanoparticle synthesis.

2.2. Experimental methods

2.2.1. Polymeric nanoparticle synthesis

The production of biodegradable PLGA based nanoparticles (NPs) loaded with BSA-FITC started from a saturated solution that was prepared by stirring an excess of BSA-FITC in phosphate buffer (PBS, pH 7.4) at room temperature (25 °C) for 24 h. The samples were then centrifuged at 3000 rpm for 10 min, and the supernatant was subsequently filtered through a nylon membrane (pore size 0.45 μm) as previously described (Rescignano et al., 2013).

The double emulsion (water/oil/water) method (Rescignano et al., 2012) with subsequent solvent evaporation was used to obtain NPs loaded with protein. The PLGA NPs were suspended in deionised water after several washes and centrifuges. Briefly, 0.25 g of biodegradable polymer (PLGA) was dissolved in 5 mL of chloroform for 2 h under magnetic agitation. This solution was emulsified with 2 mL of PBS with BSA-FITC by using the tip sonicator (Vibracell 75043, 750W, Bioblock Scientific) for 15 min. The resulting emulsion was mixed with 2 wt.%/v of PVA aqueous solution for 30 min under sonication for the formation of a second emulsion. For the solvent evaporation, the second emulsion was transferred in 200 mL of 0.2 wt.%/v of PVA aqueous solution and it was magnetically stirred over night at room temperature. The nanoparticles were then collected by centrifugation at 5500 × *g* for 20 min and were washed four times with deionised water (Lee, Chen, Iruela-Arispe, Wu, & Dunn, 2007; Rescignano, Amelia, Credi, Kenny, & Armentano, 2012; Rescignano et al., 2013).

The morphology of the polymeric nanoparticles was analyzed by field emission scanning electron microscope (FESEM Supra 25, Zeiss) using secondary electrons. Samples were deposited onto fluorine-doped tin oxide (FTO) substrates using a drop casting method, allowing them to dry at room temperature for 24 h and they were then gold coated by an Agar automatic sputter coated.

2.2.2. Cellulose nanocrystal synthesis

Microcrystalline cellulose (MCC) was hydrolysed in sulphuric acid hydrolysis (64 wt.%/wt.) at 45 °C for 30 min as previously reported (Cranston & Gray, 2006; Fortunati, Armentano, Zhou, Iannoni, et al., 2012). After acid removal, dialysis and ultrasonic treatment, the resultant cellulose nanocrystal aqueous suspension was approximately 0.5 wt.%/wt. and the hydrolysis procedure yield was ca. 20%. Mixed bed ion exchange resin (Dowex Marathon MR-3 hydrogen and hydroxide form) was added to the cellulose suspension for 48 h and then removed by filtration. CNC were neutralized by addition of 1.0% (v/v) of 0.25 mol L^{-1} NaOH (Wang et al., 2007). The morphological investigation of obtained cellulose nanocrystals

was performed by the transmission electron microscopy (TEM, JEOL JEM-1010), using an accelerating voltage of 100 kV. One drop of CNC aqueous solution was directly placed on electron microscopic copper grids, dried at room temperature and directly observed.

2.2.3. PVA/CNC binary bio-nanocomposites

Polyvinyl alcohol (PVA) bio-nanocomposite films reinforced with cellulose nanocrystals (CNC) were prepared by solvent casting in water (Roohani et al., 2008). PVA aqueous solutions were firstly prepared. A given amount of PVA (1.5 g) was dissolved in 15 mL distilled water at 80 °C for 2 h under mechanical stirring. Then, the solution was kept under stirring to reach room temperature (RT). To obtain films with different compositions, the solution was mixed with a specific amount of the aqueous dispersion of cellulose nanocrystals and sonicated (Vibracell 75043, 750W, Bioblock Scientific) for 2 min. The resulting mixture was cast in a Teflon® substrate and placed in a 37 °C oven to evaporate water. Cellulose nanocrystal content in the final binary bio-nanocomposites were 0, 0.5, 1, 2, and 5 wt.% respect to the polymer weight and the samples were designed as PVA, PVA/0.5CNC, PVA/1CNC, PVA/2CNC and PVA/5CNC, respectively. The obtained films were 200–300 μm thick (film thickness was measured using a micrometer to ±0.001 mm).

2.2.4. PVA/NPs binary bio-nanocomposites

The PLGA NPs loaded with BSA-FITC were employed for the production of binary bio-nanocomposite films by solvent casting in water. PVA (1.5 g), was dissolved in 15 mL of distilled water at 80 °C for 2 h under mechanical stirring as describe above. A specific amount of PLGA NPs were added to the PVA to obtain a 0.5 wt.% of NPs respect to the PVA matrix and magnetically stirred for about 30 min in order to allow the dispersion in PVA. The resulting final mixture was cast in a Teflon® substrate and placed in a 37 °C oven to evaporate water. The obtained films, designed as PVA/0.5NPs, had a thickness ranging between 200 and 300 μm (film thickness was measured using a micrometer to ±0.001 mm).

2.2.5. PVA ternary bio-nanocomposites

Ternary bio-nanocomposite films reinforced with 0.5 wt.% of cellulose nanocrystals and 0.5 wt.% of PLGA NPs loaded with BSA-FITC were also prepared. PVA (1.5 g) was dissolved in 15 mL distilled water at 80 °C for 2 h under mechanical stirring as in the case of binary systems. To obtain films with 0.5 wt.%, the CNC solution was mixed with the polymer and sonicated (Vibracell 75043, 750W, Bioblock Scientific) for 2 min. The specific content of cellulose nanocrystals for the ternary system was selected on the base of the results of the PVA/CNC binary film characterization. A specific amount of PLGA NPs were added to the PVA/CNC mixture in order to obtain a 0.5 wt.% of NPs respect to the PVA matrix. The solution was magnetically stirred for about 30 min in order to allow the dispersion of the NPs in the PVA matrix and their interaction with CNC. The resulting final mixture was cast on a Teflon® substrate and placed in a 37 °C oven to evaporate the water. The obtained films, designed as PVA/0.5CNC/0.5NPs, had a thickness ranged between 200 and 300 μm (film thickness was measured using a micrometer to ±0.001 mm).

2.3. Characterization methods

The microstructure of PVA bio-nanocomposite films, binary and ternary systems, was investigated by field emission scanning electron microscope, FESEM, Supra 25-Zeiss. Cryo-cross sections of the bio-nanocomposites were sputtered with gold and then analyzed.

The mechanical behaviour of neat PVA and PVA bio-nanocomposites, binary and ternary systems, was evaluated by tensile tests, performed on rectangular probes (100 mm × 10 mm) on the basis of UNE-EN ISO 527-5 with a crosshead speed of 5 mm min^{-1}, a load cell of 500 N and an initial gauge length of 50 mm. Elongation at break (ε_b), tensile strength (σ_b) and Young's modulus (E_{Young}) were calculated from the resulting stress–strain curves. The measurements were done at room temperature and at least five samples for each formulation were tested.

Differential scanning calorimetry (DSC, Mettler Toledo 822/e) was performed from −25 to 220 °C, at 10 °C min^{-1}. Two heating and one cooling scans were performed. 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 temperature (T_m) and the crystallization temperature (T_c) were taken as the temperature of the endotherm and exothermic peaks, respectively. Three samples were used to characterize each formulation. The crystallinity degree was calculated as:

$$\chi = \frac{1}{(1 - m_f)} \left[\frac{\Delta H}{\Delta H_0} \right] \times 100 \quad (1)$$

where ΔH is the enthalpy of melting or crystallization, ΔH_0 is enthalpy of melting for a 100% crystalline PVA sample, taken as 161.6 J/g, (Roohani et al., 2008) and $(1 - m_f)$ is the weight fraction of the PVA matrix in the sample.

Thermogravimetric measurements (TGA) were performed by using a Seiko Exstar 6300. Heating scans from 30 to 900 °C at 10 °C min^{-1} in nitrogen atmosphere were performed for each formulation and three replications were done.

2.4. Biological characterization

2.4.1. Stem cell isolation and culture

Human bone marrow–mesenchymal stem cells (hBM-MSCs) were isolated and cultured as previously described elsewhere (D'Angelo et al., 2010, 2012; Martino et al., 2009). Briefly, bone marrow cells were obtained from washouts of the medullary cavities of the femurs of informed patients undergoing primary total hip replacement. Mononuclear cells were isolated according to density gradient on Lympholyte (Cedarlane Laboratories Limited) and seeded in 25 cm^2 culture flasks at a density of 2.5 × 10^6 cells mL^{-1} in control medium consisting of RPMI-1640 (Euroclone) medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM of L-glutamine, and 100 U mL^{-1} of penicillin–streptomycin (Euroclone) in a humidified atmosphere and 5% carbon dioxide (CO_2) at 37 °C. After 5–7 days, the non-adherent cells were removed, and fresh medium was added to the flasks. After 15 days, a fibroblast-like colony started to grow. The medium was changed every 3 days.

2.4.2. PLGA NPs internalization by human stem cells: binary and ternary systems

To explore if PLGA NPs were released by PVA and internalized from the stem cells, hBM-MSCs were placed on PVA/0.5CNC, PVA/0.5NPs binary bio-nanocomposites, PVA/0.5CNC/0.5NPs ternary bio-nanocomposite and PVA neat for comparison, at a starting concentration of 2 × 10^3 cells mL^{-1} of control medium. In some experiments, hBM-MSCs were seeded 24 h prior to the PVA/NPs binary administration at a density of 2 × 10^3 cells/well on glass microscope slide in a 24-well plate in 1 mL culture medium (RPMI, 10% Fetal Bovine Serum (FBS) plus Penicillin/Streptomycin, 100 U mL^{-1} e 2 mM L-glutamine).

2.4.3. Cell viability assay

hBM-MSCs (2 × 10^3 cells mL^{-1}) viability on different substrates (PVA, PVA/0.5CNC, PVA/0.5NPs PVA/0.5CNC/0.5NPs) was measured by using the XTT salt solution (Sigma) assay. At different time points (3, 7, and 14 days), cell viability was measured by assaying the mitochondrial dehydrogenase activity incubating cultures with XTT reagent for 4 h at 37 °C according to the manufacturer's

recommendations. The absorbance of the samples was measured using a microtiter plate reader (GDV) at 450 nm with a reference wavelength of 650 nm.

2.4.4. Immunofluorescence microscopy

Immunofluorescence tests were performed as previously described (Martino et al., 2009); briefly, cells were fixed in 4% paraformaldheyde for 30 min. After being washed with PBS, samples were mounted, and nuclei were counterstained with Vectashield with DAPI (Vector Laboratories Inc.), and analyzed with fluorescence microscopy Eclipse-TE2000-S, Nikon using the DAPI filter at ex λ 340–380, DM λ 400; RA λ 435–485 and FICH filter at ex | 465-495 DM |505 BA | 515-555. Images were acquired by a F-ViewII FireWire camera (Soft Imaging System, Olympus) and elaborated using the Adobe Photoshop CS2 program.

3. Results and discussion

3.1. Cellulose nanocrystals and PLGA nanoparticles

Fig. 1a shows a TEM image of cellulose nanocrystals obtained after the hydrolysis process. The CNC appear well individualized with the typical dimensions ranging from 100 to 200 nm in length and 5–10 nm in width (Fortunati, Armentano, Zhou, Iannoni, et al., 2012; Fortunati, Armentano, Zhou, Puglia, et al., 2012).

The FESEM image of PLGA NPs obtained from double emulsion technique is reported in Fig. 1b. The NPs show a spherical shape with a narrow size distribution. No aggregation phenomena after drying were observed; the diameter average is 160 nm as previously demonstrated by Rescignano et al. (2013). The encapsulation efficiency of FITC-BSA in the PLGA NPs was 65% (Rescignano et al., 2013).

3.2. Cellulose nanocrystal based binary bio-nanocomposites

PVA/CNC bio-nanocomposite films reinforced with different content (0.5, 1, 2 and 5 wt.%) of cellulose nanocrystals were prepared by solvent casting in water and the mechanical, thermal and morphological behaviour of the binary systems were investigated in order to select the optimum cellulose content to use in the successive production of the ternary system with PLGA nanoparticles, main object of this research activity. Through casting from the aqueous solution, the PVA control gave a transparent, flexible film.

The PVA/CNC systems still maintained the flexibility and the transparency of the polymer matrix (data not shown). This evidence indicates that there is very limited cellulose aggregation in PVA as a result of the high level of compatibility and interaction between the hydrophilic crystalline cellulose nanocrystals and the PVA matrix (Qua et al., 2009).

The developed bio-nanocomposites showed a short-term stability in water, and PVA neat film and PVA/CNC based binary bio-nanocomposites dissolved after 10 min in aqueous medium. We previously reported the possibility to obtained a water-stable PVA film by selecting a higher hydrolization degree of the poly(vinyl alcohol) and we studied the effects of CNC amount and type on the water diffusion kinetics through PVA bio-nanocomposite films (Fortunati et al., 2013). Therefore, according to the hydrolization degree of the selected PVA, it is possible to control the dissolution time in physiological conditions, hence modulating the release kinetic of PLGA nanoparticles, main objective of this research.

3.2.1. Mechanical response

Fig. 1c and d shows the elongation at break and the Young's modulus values for the PVA matrix and the PVA/CNC based systems. The results obtained highlight the capability of cellulose nanocrystals to increase the plastic response of the material with an increase in the elongation at break value of about 57% for PVA/0.5CNC respect to the PVA matrix. This effect was enhanced with the cellulose content and the PVA/2CNC system shows a still higher value of the elongation at break that reaches 195% (Fig. 1c). However, a decrease of the elongation at break respect to the PVA film was detected in the case of the higher content of crystals (PVA/5CNC) (Fortunati, Armentano, Iannoni, & Kenny, 2010; Fortunati, Armentano, Zhou, Iannoni, et al., 2012) suggesting the progressive loss of filler efficiency and recommending the use of a lower content of CNC.

Fig. 1d compares the Young's modulus of PVA/CNC binary systems with pure PVA film. All studied bio-nanocomposites show tensile modulus slightly higher than neat PVA. The PVA/5CNC, with the higher content of cellulose studied shows the highest modulus. The PVA/0.5CNC system has Young's modulus of about 1520 MPa with an increase of 15% respect to the PVA matrix. These results highlight the reinforcement effect of cellulose nanocrystals in the elastic region that is influenced by the CNC content.

The results of the mechanical characterization underline the capability of the lower content of cellulose (0.5 wt.%) to induce a reinforcement effect in both the elastic and plastic regions, suggesting the possible use of this content of CNC in the ternary system production.

3.2.2. Thermal characterization

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were used to investigate the effect of CNC on the thermal stability of the bio-nanocomposites and also to obtain deeper insights on the interactions between PVA matrix and the reinforcement phase.

DTG curves of neat PVA and PVA/CNC bio-nanocomposites are shown in Fig. 2a. The PVA matrix presents a multi-step decomposition process with the main peak at 323 °C and a second stage at 437 °C. The first peak can be related to the release of the acetyl groups that were transformed to acetic acid molecules and successive catalytic degradation of the main chain by in situ chain stripping at higher temperature (about 440 °C) (Peresin et al., 2010).

The DTG curves of PVA/CNC binary bio-nanocomposites show that no evident shifts occur in the maximum degradation temperatures with the presence of cellulose nanocrystals even when used at the highest loadings of 5 wt.%. This fact provides additional evidence of the good interfacial adhesion obtained between PVA matrix and the dispersed phase. However, the onset degradation temperatures of the bio-nanocomposites are slightly decreased with the addition of CNC and this effect, due to the influence of cellulose thermal behaviour, was more evident with the increase of crystal content. It was previously reported, in fact, that CNC lost nearly 50% of their mass in the 150–300 °C region followed by another 25% mass loss between 300 and 500 °C, leaving significantly higher residue, nearly 23%, at 500 °C (Fortunati, Armentano, Zhou, Iannoni, et al., 2012). Nevertheless, a less intense weight loss at lower temperature and a higher intense peak around 440 °C were detected in the case of PVA/0.5CNC and PVA/1CNC binary systems underlining an increased thermal stability for these formulations.

DSC analysis was also performed in order to investigate the glass transition, crystallization and melting phenomena of PVA and PVA/CNC bio-nanocomposites in relation to their composition. Table 1 summarizes the DSC parameters for the different formulations. The PVA heating thermograms displayed successively (graphs not shown) the glass transition temperature (T_g) and the melting endotherm peak (T_m). The PVA film exhibited a relatively large and sharp endothermic curve with a peak at around 177 °C while there is a specific heat increment at around 70 °C, corresponding to the glass-rubber transition of the polymer. During the cooling scan an exothermic crystallization is presented at around 128 °C in the PVA film. A similar trend was detected for all the binary bio-nanocomposites with different content of cellulose

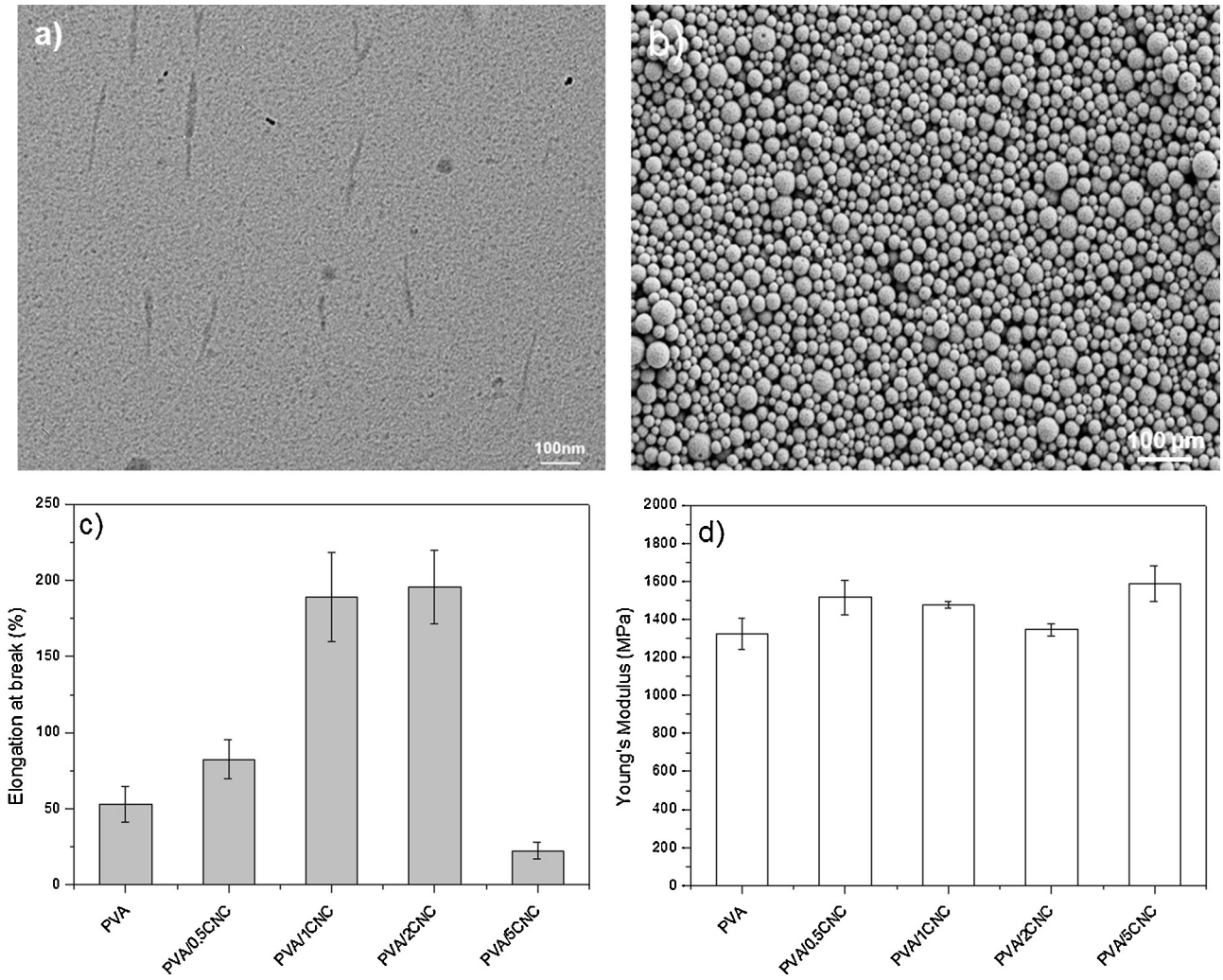

Fig. 1. TEM analysis of cellulose nanocrystal suspension (a) and FESEM image of PLGA nanoparticles (b). Elongation at break (c) and Young's modulus (d) values of PVA and PVA/CNC bio-nanocomposites.

nanocrystals. As shown in Table 1, the glass transition temperatures of the bio-nanocomposites do not change significantly respect to the polymer matrix.

The crystallinity of PVA measured during the second heating scan increases slightly with the addition of CNC and this effect is more evident at higher content of cellulose nanocrystals. However, the crystallization temperature (T_c) of the bio-nanocomposite films occurred at a higher temperature with respect to the neat PVA sample. An increase of about 13 °C was detected for PLA/0.5CNC with respect to the PVA film

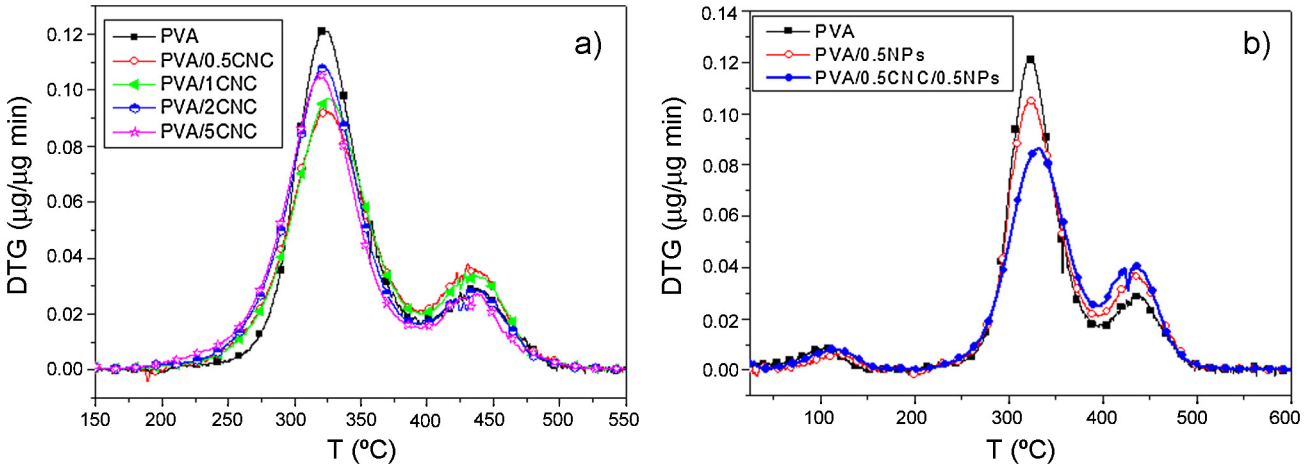

Fig. 2. DTG curves of PVA and PVA/CNC binary systems (a) and of PVA, PVA/0.5NPs and PVA/0.5CNC/0.5NPs binary and ternary systems (b).

Table 1
Thermal properties of PVA and PVA/CNC binary nanocomposites.

Samples	Cooling scan			Second heating scan			
	T_g [°C]	ΔH_c [J/g]	T_c [°C]	T_g [°C]	ΔH_c [J/g]	T_c [°C]	X_c [%]
PVA	66.3 ± 0.9	25.1 ± 3.0	127.6 ± 2.0	68.9 ± 0.1	18.3 ± 3.0	176.7 ± 3.0	11.3 ± 1.0
PVA/0.5CNC	66.1 ± 1.0	17.4 ± 0.1	140.4 ± 0.6	69.2 ± 0.2	19.8 ± 3.0	179.7 ± 0.1	12.3 ± 1.0
PVA/1CNC	66.2 ± 0.2	21.0 ± 0.8	145.0 ± 1.0	69.8 ± 0.4	20.7 ± 0.2	180.7 ± 1.0	13.0 ± 0.5
PVA/2CNC	64.6 ± 0.5	23.1 ± 0.4	156.9 ± 0.1	68.3 ± 0.1	23.2 ± 1.0	187.4 ± 0.7	14.7 ± 1.0
PVA/5CNC	63.6 ± 1.0	27.4 ± 2.0	159.9 ± 0.2	68.3 ± 0.1	30.7 ± 2.0	192.7 ± 0.1	20.1 ± 1.0

(T_c = 127.6 ± 2.0 °C). The T_c of the bio-nanocomposites increased with the CNC content reaching a value of 159.9 ± 0.2 °C in the case of the PVA/5CNC formulation. This result clearly evidences that CNC are able to promote the crystallization of the PVA matrix, acting as heterogeneous nucleating agents. A similar trend was detected also for the melting temperature during the heating scan with an increase in the T_m value of about 16 °C in the PVA/5CNC sample. Both the melting point and the crystallinity degree increased as a function of the CNC content confirming the nucleation effect of the cellulose nanocrystals and underlining the well interaction between the reinforcement phase and the PVA chains by hydrogen bonding forces.

3.2.3. Morphological behaviour

Good dispersion of cellulose in the polymer matrix is a key factor for achieving a good reinforcement. When dispersed in water, CNC form a very stable suspension due to the strong hydrophilic characteristics of cellulose. Bio-nanocomposite films with different loadings of CNC were prepared by careful mixing of PVA water solution and CNC suspension followed by film casting and controlled water evaporation. The mixing time was carefully selected in order to guarantee the well interaction between PVA molecules and the crystals. The transparency of bio-nanocomposite films confirms the homogeneous dispersion of CNC in PVA.

Further evidence of the uniformity of the dispersion is obtained from the fractured surface of PVA/CNC systems. The morphology of the binary bio-nanocomposite films was characterized by FESEM. Fig. 3 shows the fractured surface of the unfilled partially hydrolysed PVA matrix (Fig. 3a and b) and the related bio-nanocomposite films reinforced with 0.5, 1, 2 and 5 wt.% of CNC (Fig. 3c–h), just after fracture. The fractured surface of the unfilled films of PVA is smooth and uniform. No clear evidences of the CNC presence is observed in the bio-nanocomposite films. This is due to the nanoscale of the filler, but it also indicates that no aggregates are present in the films and that the dispersion of the CNC within the polymeric matrix is rather good (Roohani et al., 2008). However, the aspect of the fractured surface changes with the filler concentration. In fact, the fractured surface roughness increases being more evident in the case of PVA/5CNC system loaded with the highest content of cellulose analyzed (Roohani et al., 2008).

3.3. PLGA nanoparticle based bio-nanocomposites: binary and ternary systems

Ternary systems reinforced with 0.5 wt.% of cellulose nanocrystals (the content was selected on the basis of the PVA/CNC binary system characterization) and 0.5 wt.% of PLGA polymeric nanoparticles were also prepared by solvent casting in water and their mechanical, thermal and morphological behaviour was investigated.

The PVA/0.5NPs binary system was also produced and characterized in order to understand the effect of biopolymeric nanoparticles in PVA and compare this behaviour with the ternary film. PVA/0.5NPs and PVA/0.5CNC/0.5NPs maintained the flexibility and the transparency of the polymer matrix (data not shown). This fact as described below indicated that no aggregation phenomena occur during the bio-nanocomposite production. The developed bio-nanocomposites show a short-term stability in water; a dissolution time of 10 min for PVA/0.5NPs binary film was measured while a more stable behaviour was detected for PVA/0.5CNC/0.5NPs ternary bio-nanocomposite that reached a completely dissolution after 45 min in water at 37 °C.

3.3.1. Mechanical response

The mechanical behaviour of PVA, PVA/0.5NPs and PVA/0.5CNC binary systems, and PVA/0.5CNC/0.5NPs ternary films, was compared in order to understand the synergic effect of polymeric nanoparticles and cellulose crystals on the tensile response of the PVA matrix. The results of tensile tests are summarized in Table 2 and the data on the PVA/0.5CNC system, previously discussed, are reported here for comparison.

The results for PVA/0.5NPs binary system underlined the effect of PLGA polymeric nanoparticles as plasticizer agent in the PVA film. A great increase of elongation at break was, in fact, detected for PVA/0.5NPs with respect to the PVA matrix with a consequent evident decrease in the Young's modulus. The presence of surfactant (PVA) on the surface of PLGA polymeric nanoparticles favours the interactions with the adjacent chains of PVA matrix that allow the relevant plastic response of the bio-nanocomposite systems.

The PVA/0.5CNC/0.5NPs ternary system showed an improvement of Young's modulus (1240 ± 40 MPa) respect to the PVA/0.5NPs (150 ± 20 MPa) binary system. This fact is due to the presence of CNC, like previously discussed, able to act as reinforcement agents for the elastic response of the PVA matrix. The increase in the elastic region could be ascribed to increased interactions between PVA chains and cellulose nanocrystals via hydrogen bonding. When the degree of hydrolysis of the polymeric matrix is high, the number of hydroxyl groups growths and consequently, the number of hydrogen bonds increases, as previously shown by infrared analysis (Fortunati et al., 2013). Such interactions lead to a more rigid material (Roohani et al., 2008). However, a decrease in tensile strength and elongation at break was detected in ternary system with respect to the neat polymer. The elongation at break is affected by the volume fraction of the added reinforcement, the dispersion in the matrix, and the interaction between the reinforcement and the matrix (Colom, Carrasco, Pages, & Canavate, 2003). In our case, the combined presence of cellulose nanocrystals and PLGA nanoparticles causes substantial local stress concentrations and then failure at reduced strain values. In addition, although the PLGA nanoparticles are able, when alone, to increase the plastic

Table 2
Mechanical properties of PVA and PVA binary and ternary bio-nanocomposites.

Samples	σ_b [MPa]	ε_b [%]	E_{young} [MPa]
PVA	37 ± 5	53 ± 12	1320 ± 80
Binary systems			
PVA/0.5NPs	34 ± 3	270 ± 30	150 ± 20
PVA/0.5CNC	25 ± 2	83 ± 13	1520 ± 90
Ternary system			
PVA/0.5CNC/0.5NPs	28 ± 2	22 ± 6	1240 ± 40

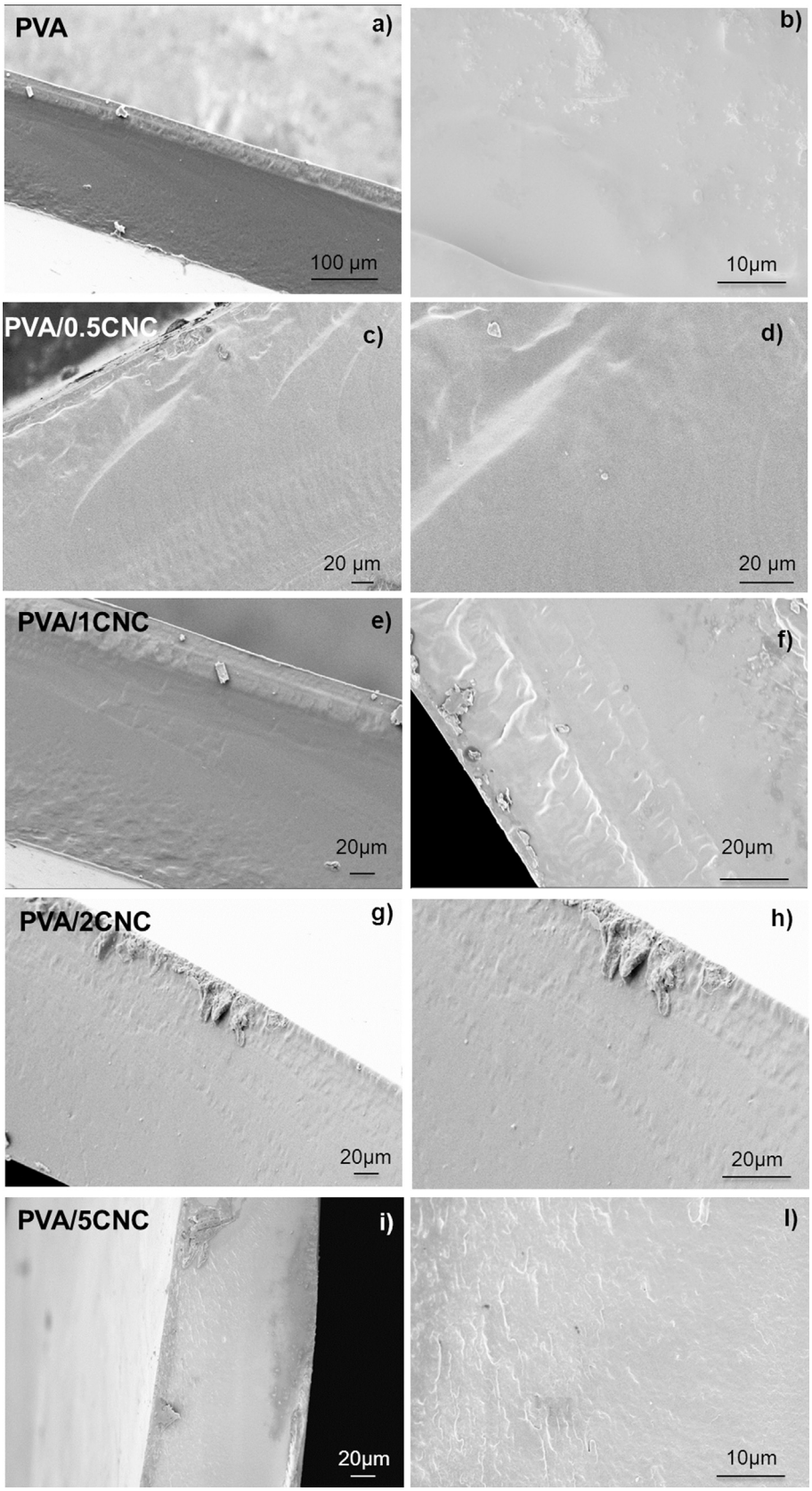

Fig. 3. Microstructure of PVA and PVA/CNC fractured surfaces.

Table 3
Thermal properties of PVA and PVA binary and ternary nanocomposites.

Samples	Cooling scan			Second heating scan			
	T_g [°C]	ΔH_c [J/g]	T_c [°C]	T_g [°C]	ΔH_c [J/g]	T_c [°C]	X_c [%]
PVA	66.3 ± 0.9	25.1 ± 3.0	127.6 ± 2.0	68.9 ± 0.1	18.3 ± 3.0	176.7 ± 3.0	11.3 ± 1.0
Binary systems							
PVA/0.5NPs	62 ± 4	27.4 ± 1.5	149.2 ± 3.0	63.8 ± 2.6	21.0 ± 1.7	187.4 ± 1.1	13.0 ± 1.0
PVA/0.5CNC	66.1 ± 1.0	17.4 ± 0.1	140.4 ± 0.6	69.2 ± 0.2	19.8 ± 3.0	179.7 ± 0.1	12.3 ± 1.0
Ternary system							
PVA/0.5CNC/0.5NPs	64.9 ± 0.9	30.9 ± 3.0	157.5 ± 1.2	68.0 ± 1.2	38.7 ± 2.7	189.2 ± 1.5	23.1 ± 2.9

response of the system, probably the interfacial adhesion with the PVA was not enhanced by the presence of cellulose nanocrystals (Pei, Zhou, & Berglund, 2010).

The results obtained demonstrate the possibility to modulate the mechanical response of these multifunctional systems taking into account the final application. Our idea is to create a system able to release specific drugs thanks to the presence of PLGA polymeric nanoparticles, to interact with a biological system (stem cells), and to control its mechanical properties with the presence of nanocellulose crystals. The effect, in fact, of chemico-physical, topographical and mechanical properties of a biomaterial on the induction of stem cell differentiation (D'Angelo et al., 2010; Martino, D'Angelo, Armentano, Kenny, & Orlacchio, 2012) as well as on the preservation of their stem cell status is well known in the scientific literature (D'Angelo et al., 2011; McMurray et al., 2011). Stem cells ability to convert mechanical cues on biochemical signals and in turn modulate their fate is a relatively recent acquisition of the scientific research (Engler, Sen, Sweeney, & Discher, 2006).

3.3.2. Thermal characterization

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were used to investigate the effect of CNC and PLGA nanoparticles on the thermal stability of the bio-nanocomposite systems. DTG curves of neat PVA and PVA based bio-nanocomposites are shown in Fig. 2b.

The DTG curves of PVA/0.5NPs bio-nanocomposites show the same behaviour of neat PVA curve with a multi-step degradation, the first one is at around 323 °C and a second stage at 437 °C. The curve of binary system PVA/0.5NPs shows a less intense weight loss at lower temperature and an higher intense peak around 440 °C systems underling an increased thermal stability for these nanocomposite formation. The same phenomenon, with a higher intensity, is observed for the curve of PVA/0.5CNC/0.5NPs ternary system. The ternary film presents a shift of the peak at 323 °C to a higher temperature due to the presence of cellulose material.

The thermal properties of cellulose nanocrystals and PLGA nanoparticles based PVA bio-nanocomposites were also determined from DSC thermograms.

Table 3 summarizes the values obtained by DSC analysis of the PVA film, PVA/0.5NPs and PVA/0.5CNC binary systems, and PVA/0.5CNC/0.5NPs ternary film. The data about the PVA/0.5CNC system are also reported here for comparison. The glass transition temperatures (T_g) of the PVA/0.5NPs binary bio-nanocomposite do not change significantly with respect to the polymer matrix. The T_g values, obtained in the cooling scan, change by almost 2–3 °C respect to the PVA film. However, an evident increase in

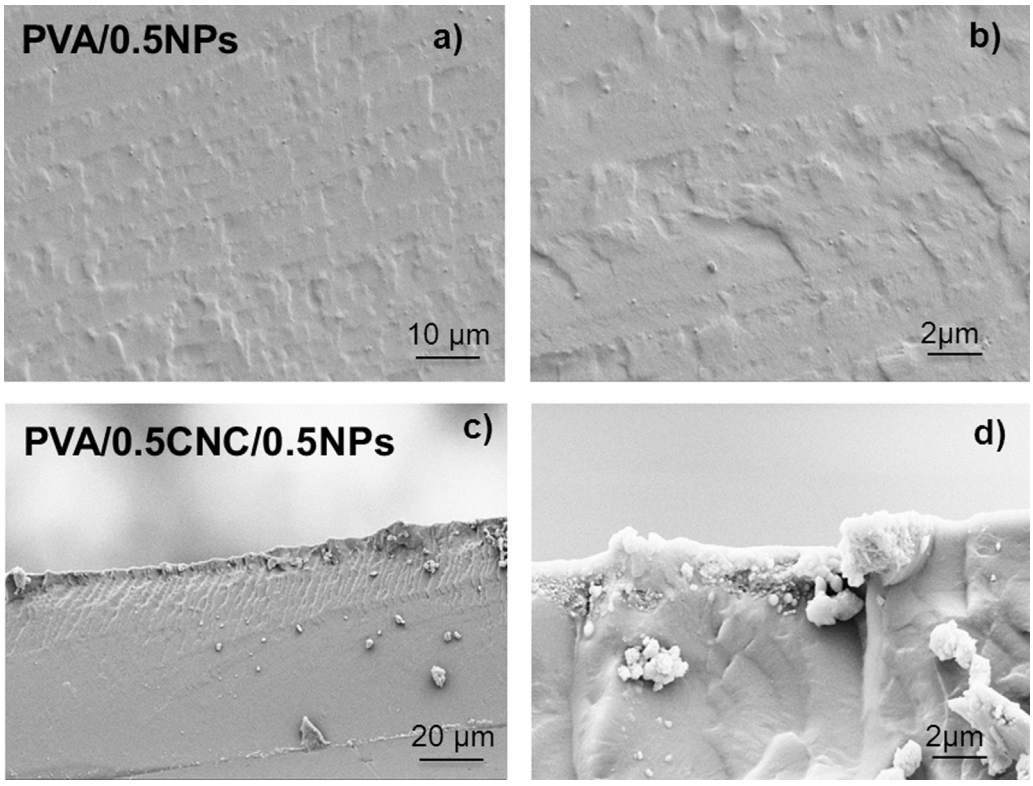

Fig. 4. Microstructure of PVA/0.5NPs and PVA/0.5CNC/0.5NPs binary and ternary system fractured surfaces.

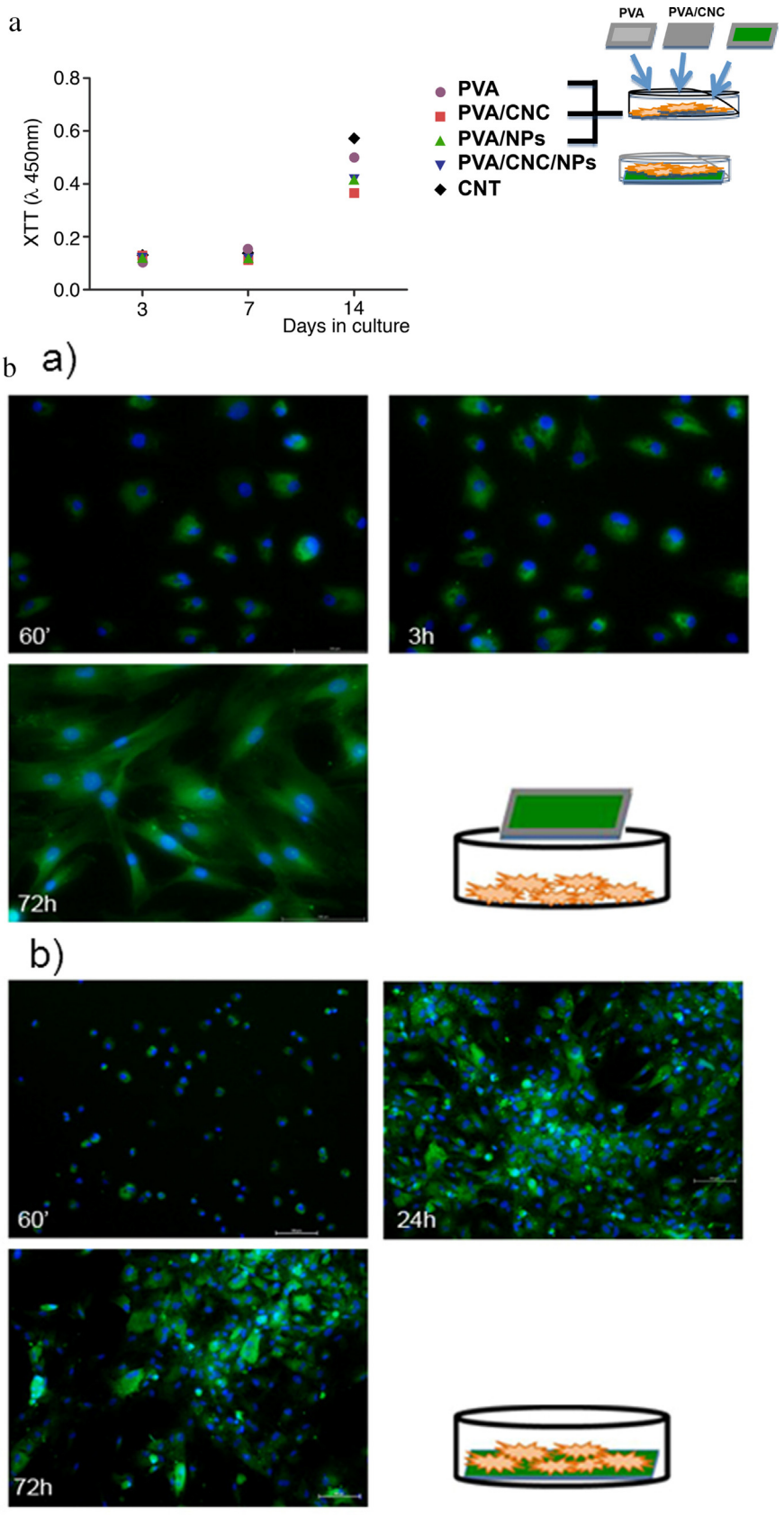

Fig. 5. Panel a: XTT assay shows the biocompatibility of PVA, PVA/0.5NPs and PVA/0.5CNC/0.5NPs binary and ternary systems towards BM-MSCs. Panel b: Up-take of NPs released by PVA/0.5NPs binary (a) and PVA/0.5CNC/0.5NPs (b) ternary systems.

the crystallization temperature (T_c) of about 22 °C was detected for PVA/0.5NPs respect to the PVA matrix. This behaviour was also detected in the case of PVA/0.5CNC binary film, highlighting the ability of cellulose to act as nucleating agent in the polymer matrix. This nucleation effect was enhanced in the case of the ternary system due to the combined effects of CNC and PLGA nanoparticles on the thermal properties of PVA. The T_c of PLA/0.5CNC/0.5NPs occurred, in fact, at 157.5 ± 1.2 °C with an increase of about 30 °C respect to PVA. Moreover, the PLA/0.5CNC/0.5NPs ternary system shows an increase of the crystallization degree that raised up from about 11% for the PVA matrix to 23% for the PVA/0.5CNC/0.5NPs bio-nanocomposite during the second heating scan. This result clearly evidences the crystallization effect induced by the synergic presence of cellulose and PLGA polymeric nanoparticles that explains also the increased elastic response and the low plastic response in the mechanical behaviour of the analyzed bio-nanocomposites. Moreover, an increase in the T_m value of about 10 °C for the PLA/0.5NPs binary systems and of 13 °C for the PLA/0.5CNC/0.5NPs sample was detected during the heating scan. This is ascribed to strong interactions between PVA and nanofillers and between adjacent PVA chains by hydrogen bonding forces and, as a consequence, highly crystalline polymers and bigger crystalline domains could be formed (Roohani et al., 2008).

3.3.3. Morphological behaviour

The morphology of PVA/0.5NPs and PVA/0.5CNC/0.5NPs films was investigated in order to study the synergic effect of NPs and CNC incorporation on the bio-nanocomposite structure. Fig. 4 shows the fractured surface of PVA/0.5NPs and PVA/0.5CNC/0.5NPs bio-nanocomposites just after cold-fracture.

On the fractured surface of the PVA/0.5NPs (Fig. 4a and b) binary film it is possible to observe the presence of PLGA nanoparticles that are well dispersed in the PVA matrix. The presence of the surfactant (PVA) on the PLGA nanoparticle surface (Rescignano et al., 2012) supports the interaction of biopolymeric nanosystem with the matrix. The PVA shell around the NPs, also influences the different physical properties of PLGA nanoparticles and their interaction with the surrounding environment including cells membrane (Boury et al., 1995; Shakesheff, Evora, Soriano, & Langer, 1997). Sahoo, Panyam, Prabha, and Labhasetwar (2002), investigated the effects of various factors that influenced the amount of PVA forming the shell around the polymeric NPs. They concluded that it is possible to control the residual PVA by altering the surfactant concentration or type of organic solvent used during NPs production (Sahoo et al., 2002). The control of this parameter is an important factor that influences the cellular uptake of biopolymeric NPs. The fracture surfaces of PVA ternary bio-nanocomposites were also investigated by FESEM (Fig. 4c and d) in order to evaluate the influence of cellulose nanocrystal and PLGA nanoparticles introduction on the microstructure. An increase in the surface roughness was detected for this system due to the presence of cellulose (Fortunati, Armentano, Zhou, Iannoni, et al., 2012), while some CNC flakes and agglomerates of nanoparticles appear embedded in the PVA matrix.

3.4. Biological evaluation

First, we tested the stability of PVA, PVA/0.5CNC, PVA/0.5NPs binary bio-nanocomposites and PVA/0.5CNC/0.5NPs ternary bio-nanocomposite within the culture medium under canonical cell culture conditions (T = 37 °C and 5% CO_2). After 10 min of incubation, PVA, of the ternary systems, is dissolved and as a consequence CNC and NPs (content of 0.5 wt.% of CNC and NPs) were delivered within the culture medium. The combined effect of hydrophobic PLGA and CNC influence the different diffusion properties, and in turn the stability in water of PVA/0.5CNC/0.5NPs ternary film that remains stable over 45 min. As a consequence, in our experimental conditions the ternary, PVA bio-nanocomposite is dissolved gradually and NPs delivered progressively. Next, we evaluated the biocompatibility of PVA, PVA/0.5CNC, PVA/0.5NPs binary bio-nanocomposites, and PVA/0.5CNC/0.5NPs ternary bio-nanocomposite on stem cells. We designed two different experimental plans for the binary and ternary bio-nanocomposites. In the first set of experiments, due to the rapid PVA dissolution time, the binary bio-nanocomposite film was added to the culture medium of stem cells seeded on a glass cover slip. Here PLGA NPs and CNC were first delivered within the culture medium and then to the cells (Fig. 5 Panel a, scheme).

In the second set of experiments, due to the longest time of PVA/0.5CNC/0.5NPs ternary film dissolution, stem cells were seeded on the PVA ternary film. Here, NPs and CNC are delivered to the cells by a direct contact of the cellular plasma membrane and the film (Fig. 5 Panel a, scheme).

Fig. 5 Panel A, shows the absence of signs of toxicity in all culture conditions indicating that the PVA polymer dissolution in culture medium does not affect the cellular viability. This emerges by the measure of the mitochondrial dehydrogenase activity that was comparable in hBM-MSCs cultured in the presence of PVA/CNC, PVA/NPs binary bio-nanocomposites and on PVA/CNC/NPs ternary films and control culture stem cells in canonical polystyrene culture tissues. The internalization time of PLGA NPs released by the PVA binary bio-nanocomposites and the PVA ternary bio-nanocomposites was then evaluated. In Fig. 5 Panel b(a) is reported the internalization by stem cells of the PLGA NPs released by the PVA binary bio-nanocomposites. NPs rapidly delivered by the PVA dissolution are fast internalized by hBM-MSCs after 15 min of incubation. Representative images indicated that up-taken NPs are extra-nuclear and have a wide distribution within the cytoplasm (Fig. 5 Panel b(a)). Fluorescent stem cell number increases with the time of incubation. Thus, above 60 min of incubation 80% of the total stem cell population were NPs-BSA-FITC positive, whereas they were totally positive after 3 and 72 h of incubation with the PVA binary film (Fig. 5 Panel b(a)). These results were recapitulated by PVA/0.5CNC/0.5NPs ternary bio-nanocomposites. Fig. 5 Panel b(b) shows representative time points of the NPs-BSA-FITC up-taken by hBM-MSCs seeded on PVA ternary bio-nanocomposites. After 1 h of seeding on the substrate almost all the stem cell population internalized the PLGA NPs indicating that they are easily released by the PVA and fast up-taken by the stem cells. Representative images revealed a fluorescence increase at 24 and 72 h of seeding of hBM-MSCs on ternary film and showed an extra-nuclear distribution of NPs with a spreading within the cytoplasm (Fig. 5 Panel b(b), representative images). Altogether these results indicated that both binary and ternary bio-nanocomposite films are suitable tools for vehiculating polymeric nanoparticles loaded with specific drugs to stem cells. Furthermore the comparable results in terms of NPs uptake (time and concentration) are independent of the deliver modality suggest that the two different PVA bio-nanocomposites could represent a good device as NPs vehicles.

4. Conclusions

In this study, we have shown the feasibility to incorporate BSA-FITC loaded PLGA NPs into PVA/CNC bio-nanocomposite films. These systems show potential for a variety of localized and fast controlled drug delivery applications. Binary and ternary bio-nanocomposites of PVA were successfully produced by the solvent casting method in water, showing a good transparency. Cellulose nanocrystals induced an increase of the Young's modulus and of the elongation at break, underlining the capability of the lower content of cellulose (0.5 wt.%) to induce a reinforcement effect in both the elastic and plastic regions. In the ternary bio-nanocomposites,

the thermal properties evidenced the crystallization effects of the synergic presence of cellulose and PLGA polymeric nanoparticles that explain also the increased elastic response and the low plastic response in the mechanical behaviour of the analyzed bio-nanocomposites.

Both binary and ternary bio-nanocomposite films are suitable vehicle of biopolymeric nanoparticles to adult bone marrow mesenchymal stem cells. By selection PVA with different hydrolization degrees we could modulate the dissolution time in culture medium, hence modulating the release kinetics of PLGA NPs.

Together these characteristics make our "nano with nano" bio-nanocomposites based on PVA, CNC and polymeric nanoparticles, suitable to be loaded with bio-macromolecules with pharmacological properties appropriate for therapeutic applications.

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