

Silk fibres grafted with 2-hydroxyethyl methacrylate (HEMA) and 4-hydroxybutyl acrylate (HBA) for biomedical applications

*Original*

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## **Abstract**

Silk fibroin may be chemically modified by grafting, with the purpose of improving its properties according to the desired function. In this study, silk fabrics from *Bombyx mori* silk fibres were grafted with 2-hydroxyethyl methacrylate (HEMA), as well as a binary mixture of HEMA and 4-hydroxybutyl acrylate (HBA). The samples were then electrospun from trifluoroacetic acid and treated with aqueous methanol. The % weight gains ascribable to HEMA and HBA were successfully determined through Raman spectroscopy. PolyHEMA made the fibres more hydrophilic and hindered crystallization into  $\beta$ -sheet only upon electrospinning and treatment with aqueous methanol; the presence of the HBA component in the grafting mixture did not further decrease the ability of silk fibroin to rearrange into  $\beta$ -sheet, due to its low contents (below 5%) under the used experimental conditions. Fibrillation partially occurred in the grafted fabrics; the electrospun samples maintained their nanostructured morphology. The surface of the substrates under investigation was compatible with cell attachment and growth, which were higher for the nanofibres. Cell adhesion and proliferation may be modulated by varying the surface chemistry and topography of the fabrics; grafting improved the surface properties of silk fibroin for enhanced functional performance in view of biomedical applications.

**Keywords:** electrospinning; vibrational spectroscopy; fibroblasts culture.

1  
2 **SILK FIBRES GRAFTED WITH 2-HYDROXYETHYL METHACRYLATE (HEMA)**  
3  
4 **AND 4-HYDROXYBUTYL ACRYLATE (HBA) FOR BIOMEDICAL**  
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6 **APPLICATIONS**  
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## 1. INTRODUCTION

Silks are fibrous proteins characterized by outstanding tensile properties and high biocompatibility, which make them useful in biomedical applications [1]. Silk fibres can be obtained from a wide variety of silkworms, such as domesticated *Bombyx mori*, and spiders [2]. The specific and remarkable properties of *B. mori* silk derive from its structure, which consists of a core containing at least two main fibroin proteins (light and heavy chains, of 25 and 325 kDa, respectively), coated by sericin, a water soluble glue-like protein. Silk fibroin as such or properly modified by chemical and enzymatic methods has gained increasing attention in biomedical applications, due to its environmental stability, outstanding mechanical properties, controlled proteolytic biodegradation, and morphologic flexibility [1-10].

In this context, electrospinning has proved a very useful technique to extend silk fibroin applications, since it allows to obtain fibres characterized by a porous structure and diameters ranging between nanoscale and microscale orders [8,9]; the electrospun nanofibres have been used as substrates for drug delivery systems, wound dressing and tissue engineering [1,2,6,7,10].

Among the chemical methods exploited to modify the silk fibre surface, grafting proved to be a superior way to attain long-lasting modifications due to covalent bond formation. With the purpose of improving the textile properties of silk, the graft copolymerization of vinyl monomers onto silk has been introduced in Japan in the early 1960s and has been widely applied in the textile industry [11]. The graft copolymerization utilizes grafting agents that enable to modify the silk properties depending on the grafting extent, the molecular weight of the grafted polymer as well as its functional groups [12-14]. In particular, 2-hydroxyethyl methacrylate (HEMA) and 4-hydroxybutyl acrylate (HBA) have been used to modify silk due to their peculiarity of increasing comfort and easy-care properties, while preserving handle and drape [15,16]. On the other hand, polyHEMA has been used since the early 1960s in

121  
122 several biomedical applications (i.e. as component of dental adhesives, in soft contact lenses,  
123 vascular grafts, soft tissue substitutes and drug delivery systems) [17-20], thanks to its  
124 biocompatibility, high permeability to small metabolites, high hydrophilicity and resistance to  
125 adhesion of blood proteins and cells. Also polyHBA has been proposed as hydrogel [21].  
126 HBA has been used as copolymer building block for biomedical applications [22], such as  
127 tissue engineering [23] and drug delivery systems [24].

135 In the light of these findings, we tested the possibility of using HEMA- and HBA-grafted silk  
136 fibroin (designed for textile purposes) also for biomedical applications, as scaffold for soft  
137 tissue engineering. HEMA and HBA have a hydrophilic OH group at the end of their side  
138 chain, which is slightly longer in the latter. The balance of hydrophilicity and hydrophobicity  
139 of the surface of silk fibroin may be modified by varying the HEMA and HBA contents,  
140 which is useful in the design of biomaterials for tissue engineering. In this context, Dhyani  
141 and Singh [25] have reported that the graft polymerization of polyHEMA on *B. mori* silk  
142 fibroin films is a good strategy able to improve cell proliferation and adhesion in view of  
143 tissue engineering applications.

154 In a previous study [26], *B. mori* silk fabrics were successfully modified by grafting with  
155 HEMA and a binary mixture of HEMA and HBA; the grafted silk and pure control silk  
156 fabrics were solubilized in trifluoroacetic acid (TFA), electrospun and subsequently immersed  
157 in aqueous methanol, to induce the rearrangement of the as-electrospun nanofibres (mainly  
158 characterized by unordered/Silk I conformations) [27] towards an insoluble  $\beta$ -sheet structure  
159 [28]. Preliminary vibrational analyses of the obtained devices allowed to clarify the role  
160 played by the grafted polymer on the silk conformation [26].

169 In the present study, a more complete vibrational characterization was carried out and the  
170 samples were further assessed by SEM imaging, in view of their possible applications as  
171 biomedical devices. Contact angle measurements were carried out to evaluate the possible  
172 surface hydrophilicity changes induced by grafting; this information was correlated with

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182 vibrational data. NIH 3T3 fibroblast cell line was employed to study the biological response  
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184 of the substrates, with the aim to demonstrate their suitability for possible biomedical  
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186 applications. To the best of our knowledge, our work represents the first study on the  
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188 biocompatibility of silk fabrics and nanofibres grafted with HEMA and HEMA/HBA.  
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## 192 2. MATERIALS AND METHODS

### 195 2.1 Materials and grafting

196  
197 *B. mori* silk fabrics were dewaxed with a binary methanol/benzene (50/50 v/v) mixture at  
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199 30°C for 3 days. HEMA and HBA were purchased from Wako Pure Chemical Industries Ltd.,  
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201 Tokyo and were used without further purification.  
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203  
204 The silk fabric grafted with HEMA (indicated hereafter as HEMA35-grafted sample) was  
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206 prepared as previously reported [26], using a grafting system containing 35% owf (over  
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208 weight fibre) HEMA and 2.5% owf ammonium persulfate (APS) as initiator, at pH 3 and  
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210 80°C. At the end of the reaction, the HEMA-grafted silk fabric was extracted with acetone to  
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212 remove unreacted monomer, washed with boiling water, dried and weighed.  
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214  
215 To obtain the silk fabrics grafted with HEMA and HBA, the reaction was performed under the  
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217 same conditions, using binary mixtures containing 25% owf HEMA and 10% owf HBA (first  
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219 experiment, HEMA25\_HBA10-grafted sample) and 30% owf HEMA and 5% owf HBA  
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221 (second experiment, HEMA30\_HBA5-grafted sample). Silk fabrics grafted with HBA were  
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223 prepared for comparison [26]. The grafting system contained different amounts of HBA (i.e.  
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225 20%, 35%, 45%, 54%, 100% owf).  
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228 The percentage weight gain (w.g.) of the prepared samples was determined according to the  
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230 following equation:

$$231 \quad \% \text{ weight gain} = \frac{W_t - W_0}{W_0} \times 100 \quad (1)$$

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233  
234 where  $W_0$  was the initial weight of the dry fabric, and  $W_t$  was the weight of the fabric after  
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236 grafting, washing and drying.  
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## 2.2 Electrospinning and aqueous methanol treatment

The control silk fabric, the HEMA35-grafted, HEMA25\_HBA10-grafted and HEMA30\_HBA5-grafted fabrics were electrospun from trifluoroacetic acid (TFA, Wako Pure Chemical Industries Ltd. Tokyo, Japan, used without further purification) at 8 wt% concentration, under the experimental conditions previously reported [26].

All the nanofibres were immersed in 50% v/v aqueous methanol solution for 10 minutes, rinsed with water and allowed to dry at room temperature for 12 h.

## 2.3 Raman and IR vibrational spectroscopy

Raman spectra were recorded on a Bruker MultiRam FT-Raman spectrometer equipped with a cooled Ge-diode detector. The excitation source was a Nd<sup>3+</sup>-YAG laser (1064 nm) in the backscattering (180°) configuration. The focused laser beam diameter was about 100 μm and the spectral resolution 4 cm<sup>-1</sup>. Laser power at the sample was about 40 mW.

The Raman Amide I region was analyzed by a curve-fitting procedure to evaluate the content of secondary structures. **A 2-points linear baseline correction in the 1750–1580 cm<sup>-1</sup> spectral range was made prior to this procedure.** The curve-fitting analysis was performed using the OPUS version 6.5 program (Bruker Optik GmbH), which uses the Levenberg–Marquardt algorithm.

Band fitting was performed according to Lefèvre *et al.* [29] with five amide I components located at about 1640, 1656, 1667 cm<sup>-1</sup> (assigned to unordered conformations, 3<sub>1</sub> helices and β-sheet, respectively [29]), 1680 and 1695 cm<sup>-1</sup> (assigned to β-turns [29]), and two bands at about 1597 and 1615 cm<sup>-1</sup> associated with side-chain vibrations of aromatic residues. In addition, bands ascribable to the grafted polymers or protonated glutamate and aspartate residues were used when necessary.

The Raman component profiles were described as a linear combination of Lorentzian and Gaussian functions; the band widths and the percentages of the Lorentzian character were fixed according to Lefèvre *et al.* [29]. The area of each component divided by the sum of the

301 area of all amide I components was used for the determination of each secondary structure  
302 content.  
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306 IR spectra were recorded on a Nicolet 5700 FTIR spectrometer, equipped with a Smart Orbit  
307 diamond ATR accessory and a DTGS detector; the spectral resolution was 4 cm<sup>-1</sup> with 64  
308 scans for each spectrum. The ATR technique allows to investigate the surface skin of the  
309 specimens (under the used experimental conditions, the investigated sample thickness was  
310 about 2 μm). Both IR and Raman spectra were recorded in triplicate.  
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#### 313 **2.4 Morphological analysis: Scanning Electron Microscopy**

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315 The morphology of silk fabrics and silk nanofibres before and after methanol treatment was  
316 evaluated through Scanning Electron Microscopy (SEM). Samples were coated with a thin  
317 gold layer using Agar Auto Sputter Coater. Then the samples were analysed through SEM  
318 LEO – 1430 (Zeiss) equipment at different magnifications (500×, 1000×, 2000× and 5000×).  
319 The average fiber diameter size of the samples was calculated using ImageJ software. Results  
320 were reported as average value ± standard deviation (SD).  
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#### 332 **2.5 Contact angle measurements**

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334 Static contact angles were measured at room temperature using a CAM 200 Instrument (KSV  
335 NIMA, Biolin Scientific, Finland) equipped with an Attention Theta software (Biolin  
336 Scientific, Finland) for data acquisition. Three Milli-Q water droplets (volume 5 μL) were  
337 **deposited** on each sample and data were collected thereafter for 10 s with a time frame of 10  
338 ms. The data were expressed as mean value ± SD. Statistical analysis was carried out using  
339 single-factor analysis of variance (ANOVA). Values of P < 0.05 were considered statistically  
340 significant.  
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#### 349 **2.6 Cell culture and vitality assessment**

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351 Murine fibroblasts NIH 3T3 cells were obtained from American Type Culture Collection  
352 (Cat. no. ATCC CRL-1658, <http://www.lgcstandards-atcc.org>). Cells were cultured at 37°C,  
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362 in a humidified atmosphere with 5% CO<sub>2</sub>, in DMEM with 10% fetal calf serum (FBS) and 1%  
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364 antibiotic mixture (Sigma-Aldrich, Milan, Italy).  
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366 The fabrics and the methanol-treated nanofibrous scaffolds were sterilized by UV irradiation  
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368 for 1 hour. Cells were incubated at standard culture conditions on all the samples for 1 and 3  
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370 days.  
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373 The number of viable cells was estimated with calcein-AM assay. **The Calcein-AM kit**  
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375 **provides a simple, rapid, and accurate method to measure cell viability and/or cytotoxicity.**  
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377 **Calcein-AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live**  
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379 **cells. The hydrolysis of Calcein-AM by intracellular esterases produces calcein, a hydrophilic,**  
380  
381 **strongly fluorescent compound that is well-retained in the cell cytoplasm.**  
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383 For calcein-AM assays, cells were settled overnight in 96-well plates (8,000 cells/well) and  
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385 then incubated with the samples under study (i.e. fabrics and nanofibres). After incubation,  
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387 cells were washed with PBS, and then incubated for 30 min at 37°C with 2.5 μM calcein-  
388  
389 acetoxymethylester (calcein-AM) in PBS. Plates were read in an Infinite 200 Pro plate reader  
390  
391 (Tecan, Wien, Austria). **The fluorescence was read using a 485 nm excitation filter and a 535**  
392  
393 **nm emission filter [30].** Data were analyzed by ANOVA and the post hoc Tukey's test, using  
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395 the InStat software package (GraphPad Software, Inc, San Diego, CA).  
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### 400 **3. RESULTS**

#### 401 **3.1 Spectroscopic characterization: fabrics**

402  
403 Fig. 1 (A) and Fig. S1, Supplementary Material (SM), show the Raman and IR spectra of  
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405 HEMA35-grafted, HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted silk fabrics (w.g.  
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407 26%, 24% and 20%, respectively). The spectra of control silk fabric and polyHEMA have  
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409 been previously reported [26] **and are here shown for comparison.**  
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412  
413 Upon grafting, both Raman and IR spectra showed spectral features ascribable to polyHEMA  
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415 [26, 31-33] **(indicated with asterisks in Fig. 1 (A) and Fig. S1, SM).**  
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421  
422 To investigate the effective incorporation of the HBA component into the samples grafted  
423 with binary mixtures of HEMA and HBA, the marker bands due to the HBA-grafting were  
424 identified by inspecting the Raman and IR spectra of the HBA-grafted samples (with weight  
425 gains ranging between 9.7% and 57%). As can be seen from Fig. S2 and S3, SM, bands  
426 assignable to the HBA component were observed with increasing intensity at increasing  
427 weight gain at 1728, 1451, 1300, 1266, 1043, 829, 810  $\text{cm}^{-1}$  (Raman) and 1729, 1700, 1434,  
428 1398, 1260, 1160, 1061, 1038, 996 and 940  $\text{cm}^{-1}$  (IR).

437 The Raman and IR spectra of HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted  
438 fabrics (Fig. 1 and Fig. S1, SM) showed that also the HBA component was incorporated into  
439 the fabric, as shown by the appearance of its above marker bands at 1300 and 1043  $\text{cm}^{-1}$   
440 (Raman) and 1160 and 1040  $\text{cm}^{-1}$  (IR).

445 The Raman  $I_{602}/I_{644}$  and  $I_{1300}/I_{644}$  intensity ratios were identified as useful spectral markers to  
446 obtain quantitative information on the HEMA and HBA incorporation, respectively. The band  
447 at 602  $\text{cm}^{-1}$  was chosen since it was the strongest polyHEMA band falling in a spectral range  
448 free from silk fibroin bands (see Fig. 1 (A)). An analogous reason motivates the choice of the  
449 band at 1300  $\text{cm}^{-1}$  for the quantification of the HBA incorporation: this is the most intense  
450 spectral feature falling in a region relatively free from silk fibroin bands (see Fig. S2, SM).  
451 Both the  $I_{602}/I_{644}$  and  $I_{1300}/I_{644}$  ratios were found to well correlate with the sample weight gain  
452 in samples grafted with only HEMA or HBA ( $R^2$  values of 0.9735 and 0.9886, respectively,  
453 Fig. S4, SM). The plot reported in Fig. S4 (A), SM, shows the trend of the  $I_{602}/I_{644}$  intensity  
454 ratio as a function of % weight gain as calculated from the Raman spectrum of the HEMA35-  
455 grafted fabric (Fig. 1 (A)) as well as from data reported in the literature on other HEMA-  
456 grafted samples (with weight gains of 18.1% and 47.7%) [12]. Fig. S4 (B), SM, shows the  
457 trend of the  $I_{1300}/I_{644}$  intensity ratio (calculated from the spectra reported in Fig. S2, SM) as a  
458 function of the % weight gain. The good  $R^2$  values obtained for the lines reported in Fig. S4  
459 (A) and (B), SM, allowed their use for the quantitative determination of HEMA and HBA

481 incorporation into the HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted fabrics. The  
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483  $I_{602}/I_{644}$  and  $I_{1300}/I_{644}$  intensity ratios were calculated from their Raman spectra (Fig. 1 (A))  
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485 and these values were used to calculate the weight gains corresponding to the single HEMA  
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487 and HBA components (Fig. 1 (B)), by interpolation from the lines reported in Fig. S4 (A) and  
488  
489 (B), SM.  
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491  
492 The possible occurrence of changes in silk fibroin conformation upon grafting has been  
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494 investigated. The spectra reported in Fig. 1 (A) and Fig. S1, SM, showed that all the grafted  
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496 samples had a prevailing  $\beta$ -sheet conformation, as shown by the Raman bands at about 1664  
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498  $\text{cm}^{-1}$  (Amide I), 1229  $\text{cm}^{-1}$  (Amide III), 1162, 1084, 975 and 882  $\text{cm}^{-1}$  (CC stretching, CC  
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500 skeletal stretching,  $\text{CH}_3$  rocking and  $\text{CH}_2$  rocking of a  $\beta$ -sheet conformations, respectively  
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502 [34-36]), and by the IR bands at 1698-1618  $\text{cm}^{-1}$  (Amide I), 1514  $\text{cm}^{-1}$  (Amide II), 1264-1226  
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504  $\text{cm}^{-1}$  (Amide III) [37-39], 997 and 974  $\text{cm}^{-1}$  (Ala-Gly sequences in  $\beta$ -sheet conformation  
505  
506 [37]), although in some of these spectral ranges the contribution of polyHEMA was revealed.  
507  
508 The full-width at half maximum of the Raman Amide I band (FWHM) was nearly the same in  
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510 the spectra of all the fabrics, as shown in Fig. 2 (A).  
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513 To gain information on the secondary structure distribution, the Raman Amide I of the  
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515 samples under study was fitted into their components (see Fig. S5, SM, for some examples),  
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517 as detailed in the Experimental section. Amide I band fitting data (Fig. 2(B)) showed that the  
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519 HEMA35, HEMA30\_HBA5 and HEMA25\_HBA10-grafted fabrics had secondary structure  
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521 contents not significantly different from those of the silk control sample and confirmed that  
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523 upon grafting the prevailing conformation remained  $\beta$ -sheet. It may be noted that the  
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525 secondary structure distribution obtained for the silk control fabric was in good agreement  
526  
527 with the data reported by Lefèvre *et al.* [29].  
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531 An analogous conformational study was carried out on the spectra of the HBA-grafted  
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533 samples reported in Fig. S2 and S3, SM. The shift of the Raman Amide I band towards higher  
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535 wavenumber values, the weakening of the above mentioned marker bands of  $\beta$ -sheet  
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541 conformation (Fig. S2, SM), and the appearance of an IR Amide II component at 1540 cm<sup>-1</sup>  
542 (Fig. S3, SM) revealed that silk fibroin underwent conformational rearrangements upon  
543 grafting with HBA. The Raman Amide I fitting data (Fig. S6, SM) showed that at a weight  
544 gain of 9.7% the silk fibroin underwent significant changes in  $\beta$ -sheet and  $\beta$ -turns  
545 conformations, which persisted at nearly the same extents at higher weight gains.  
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548 To **separately** investigate the relative effects of the HEMA and HBA grafting on silk fibroin  
549 conformational rearrangements, the Raman spectra of HEMA-grafted and HBA-grafted  
550 fabrics with similar weight gains (i.e. 26% and 25%, respectively) were compared; as shown  
551 in Fig. 3 (A), the HBA-grafted sample showed a broader Amide I band, shifted to higher  
552 wavenumber values compared to the HEMA-grafted sample. The band fitting data (Fig. 3  
553 (B)) showed that the former had a lower content of  $\beta$ -sheet and higher amounts of  $\beta$ -turns.  
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### 556 **3.2 Spectroscopic characterization: nanofibres after immersion in aqueous methanol**

557 Fig. 4 shows the Raman and IR spectra of electrospun HEMA35-grafted, HEMA30\_HBA5-  
558 grafted and HEMA25\_HBA10-grafted nanofibres after immersion in aqueous methanol. The  
559 spectra of control silk nanofibres treated under the same conditions have been previously  
560 reported [26] **and are here shown for comparison.**  
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563 The spectra of all the samples showed the above mentioned Raman and IR marker bands of  $\beta$ -  
564 sheet conformation. However, some spectral features revealed that the nanofibres were  
565 conformationally more heterogeneous than the corresponding fabrics from which they were  
566 obtained. In fact, as shown in Fig. 2 (A), the full-width at half maximum (FWHM) of the  
567 Raman Amide I band increased in all the samples upon electrospinning and subsequent  
568 immersion in aqueous methanol. The band fitting data reported in Fig. 2 (B) showed that upon  
569 these treatments, the samples underwent significant increases in the contents of  $\beta$ -turns and  
570 unordered conformations, at the expenses of  $\beta$ -sheet, whose amount decreased.  
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573 The higher structural heterogeneity of the nanofibres with respect to the corresponding fabrics  
574 was confirmed by the IR spectra (Fig. 4 (B)), which showed for all the samples bands  
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601 assignable to Silk I [40] at about 1416, 1370, 1335 and 1019  $\text{cm}^{-1}$ , together with those of  $\beta$ -  
602 sheet and unordered conformations [41].  
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605  
606 It is interesting to note that the band at about 1725  $\text{cm}^{-1}$ , which appeared in both IR and  
607 Raman spectra (Fig. 4) of the grafted nanofibres, appeared also in control silk sample [26];  
608 therefore, besides to the grafted polymer, it is also assignable to the C=O stretching mode of  
609 the COOH group of aspartic and glutamic acids. These residues, according to previous studies  
610 [26,42], undergo protonation upon dissolution in TFA, since the  $\text{pK}_a$  value of this solvent is  
611 significantly lower than the  $\text{pK}_a$  values of the carboxylic groups of the amino acids ( $\text{pK}_a =$   
612 0.2 versus 3.7 and 4.3 for aspartic and glutamic acids, respectively).  
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### 621 **3.3 Morphological analysis**

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623 Fig. 5 reports the SEM images of the fabrics under study as well as of the nanofibres before  
624 and after aqueous methanol treatment. The images of the control silk fabric show a smooth  
625 surface, whereas the grafted silk fabrics show the occurrence of fibrillation, due to the  
626 grafting treatment. The average fibre diameter in silk fabrics was not affected by the grafting  
627 treatment, as shown in Fig. S7, SM, and was about 12  $\mu\text{m}$  in all the samples.  
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633 The membranes obtained by electrospinning of pure and grafted silk samples were free of  
634 defects and homogenous in morphology. The average diameter of the as-electrospun fibres  
635 was about 300 nm (Fig. S7, SM). After aqueous methanol treatment, all the analyzed  
636 nanofibrous membranes maintained their fibrous structure and the average fibre diameters  
637 slightly increased up to  $\sim 500$  nm (Fig. S7, SM). Moreover, the electrospun scaffolds, before  
638 and after aqueous methanol treatment, showed a quite homogeneous diameter distribution,  
639 which was comprised between 100 and 500 nm (Fig. S7, SM).  
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649 The analysis of the pore dimension distribution (Fig. S8, SM) showed that the pore size  
650 distribution did not change after the aqueous methanol treatment.  
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### 653 **3.4 Contact angle measurements**

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655 Contact angle values, measured through static contact angle equipment, are shown in Fig. 6  
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662 (A).  
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664 In general, fabric samples showed lower wettability if compared to the electrospun samples.  
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666 HEMA grafting significantly affected the surface contact angle; going from control silk to the  
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668 HEMA35-grafted samples, its value decreased from  $128^\circ \pm 2$  to  $112^\circ \pm 4$  in the fabrics, and  
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670 from  $104^\circ \pm 3$  to  $81^\circ \pm 3$  in the nanofibres. Upon adding HBA in the grafting mixture (i.e. in  
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672 the HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted samples), the contact angle was  
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674 found to slightly increase with respect to the HEMA35-grafted samples.  
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677 As shown in Fig. 6 (B), the contact angle value was found to well correlate with the % HEMA  
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679 weight gain (determined by weight measurements for HEMA35-grafted samples and by  
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681 Raman spectroscopy for HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted samples,  
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683 data reported in Fig. 1 (B)).  
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### 685 **3.5 Cell viability of fibroblasts**

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687 Fig. 7 shows the cellular viability determined by calcein-AM assay for NIH 3T3 fibroblasts  
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689 cultured on to different materials (i.e. silk, HEMA35-grafted, HEMA30\_HBA5-grafted and  
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691 HEMA25\_HBA10-grafted silk fabrics and methanol-treated nanofibres) for 1 and 3 days  
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693 culture time.  
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696 As shown in Fig. 7 (A), after 1 day, most fabrics allowed a cell growth rate similar to that of  
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698 the cells in the culture well; the HEMA25\_HBA10-grafted silk fabric was the only material  
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700 that induced a significant improvement in cell proliferation. After 3 days, the growth of cells  
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702 on the fabrics markedly decreased on HEMA35 and HEMA30\_HBA5-grafted silk fabrics  
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704 while, in the case of HEMA25\_HBA10-grafted silk fabric, the fibroblasts partly lost their  
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706 potential growth observed at 1 day, although they still showed a higher proliferation than on  
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708 the control silk fabric.  
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711 The same experiment was carried out on the electrospun nanofibres treated with aqueous  
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713 methanol; the data reported in Fig. 7 (B) show that after 3 days, the nanofibrous membranes  
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715 behaved better than the corresponding fabrics at the same culture time.  
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#### 4. DISCUSSION

The vibrational spectra of the grafted fabrics (Fig. 1 and Fig. S1, SM) showed the effective incorporation of the polymers. It must be stressed that in the IR spectra of the grafted samples (Fig. S1, SM), the silk fibroin bands were detected together with those assignable to the polymer, suggesting that the polymer is present on the surface of the fibres and its thickness is lower than the thickness analyzable by ATR/IR spectroscopy (i.e. 2  $\mu\text{m}$ , see Experimental Section).

As previously reported [26], polymeric and silk fibroin components interact each other and are not phase separated and bonds stronger than a simple physical interaction should be present [12]. According to the literature, the residues potentially involved in these covalent interactions could be serine (through its OH group) [43-45] and glycine (through its NH group) [46].

The Raman  $I_{602}/I_{644}$  and  $I_{1300}/I_{644}$  intensity ratios proved suitable to gain relative quantitative information on the HEMA and HBA incorporation, respectively, since their trend well correlated with the % weight gain due to these components in HEMA-grafted and HBA-grafted samples (Fig. S4 (A) and (B), SM); the tyrosine band at 644  $\text{cm}^{-1}$  [47] was used as internal standard. These ratios allowed to calculate the % weight gains ascribable to the single polymeric components in the HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted fabrics; the data reported in Fig. 1 (B) suggest that only a small fraction of the added HBA efficiently grafted to the fabric. This is not unexpected since methacrylic acid derivatives have been found to react with a higher efficiency than the corresponding acrylic acid derivatives [12,15]. In this light, it is not surprising that the weight gain % of the samples under study linearly increased with the percentage owf of HEMA in the grafting mixture (Fig. S9, SM), strengthening the idea that this component was the main responsible for fibre weight increase.

781  
782 The quantitative data reported in Fig. 2 showed that the secondary structure distribution was  
783 nearly the same in control silk, HEMA35-grafted, HEMA30\_HBA5-grafted and  
784 HEMA25\_HBA10-grafted fabrics; under the used experimental conditions, the  $\beta$ -sheet  
785 conformation, on which the mechanical properties of silk fibroin depend [28], was kept upon  
786 grafting.  
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792 On the other hand, the Raman spectra of HEMA-grafted and HBA-grafted samples (Fig. 3  
793 (A)) with nearly the same weight gain (26% and 25%, respectively), as well as the  
794 quantitative secondary structure data obtained through Amide I band fitting (Fig. 3 (B)),  
795 showed that the grafting with HBA had a higher impact on silk fibroin conformation than the  
796 grafting with HEMA. In fact, upon the former treatment, significant increases in the contents  
797 of  $\beta$ -turns occurred at the expenses of the  $\beta$ -sheet structure, whose amount decreased. This  
798 trend, which was not observed for the HEMA-grafted fabric, may be explained by considering  
799 the higher steric hindrance of HBA. Evidently, the higher length of the aliphatic side-chain  
800 hinders the crystallization into  $\beta$ -sheet conformation. This effect, which was observed since  
801 HBA weight gains of 9.7% (Fig. S5, SM), was not detected in the HEMA30\_HBA5-grafted  
802 and HEMA25\_HBA10-grafted fabrics, whose HBA contents were significantly lower (Fig. 1  
803 (B)).  
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Vibrational spectroscopy showed that the nanofibres were conformationally more heterogeneous than the corresponding fabrics from which they were obtained. In fact, as shown in Fig. 2 (A), the full-width at half maximum (FWHM) of the Raman Amide I band increased in all the samples upon electrospinning and subsequent immersion in aqueous methanol, suggesting that the distribution of the different elements of secondary structure became wider upon these treatments. The quantitative data reported in Fig. 2 (B) showed that upon these treatments all the samples underwent significant decreases in  $\beta$ -sheet contents and significant increases in  $\beta$ -turns and unordered conformations. It is interesting to note that the changes were lower for control silk than for the other samples. This trend indicates that the

841 grafted polyHEMA component hindered the crystallization into  $\beta$ -sheet upon aqueous  
842 methanol treatment; the presence of the HBA component in the grafting mixture did not  
843 further decrease the ability of silk fibroin to rearrange into  $\beta$ -sheet upon the same treatments,  
844 due to its low contents under the used experimental conditions.  
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850 The SEM images of the control silk fabric show a smooth surface, whereas the grafted silk  
851 fabrics show the occurrence of fibrillation, due to the grafting treatment (Fig. 5). *B. mori* silk  
852 has a core-shell structure, consisting of two components: fibroin and sericin proteins [48].  
853 Sericin can be easily removed by heating [49]. Once without the protection of the sericin  
854 proteins, silk fibroin is exposed outside, and the fibril and even the microfibril inside the  
855 fibroin filament can be easily split during the fabrication process. Hence, during the grafting  
856 procedure, the fibrils of silk fibroin were easily split and pulled out to the fibre surface.  
857 Fibrillation could further accumulate on the fabrics surface forming a layer of ‘fuzz’, so-  
858 called white stripe [50].  
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870 The membranes obtained by electrospinning were free of defects and homogenous in  
871 morphology; after aqueous methanol treatment, they maintained their fibrous structure and the  
872 average fibre diameters slightly increased (Fig. S7, SM). The pore dimension distribution  
873 (Fig. S8, SM) did not change after the aqueous methanol treatment: in all the samples most  
874 pores had sizes in the 0–0.5  $\mu\text{m}$  and 0.5–1  $\mu\text{m}$  ranges. This result suggests that cells cannot  
875 penetrate into the nanofibrous substrates, in the first phases of cell proliferation, at least.  
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883 Contact angle measurements showed that the nanofibres had a higher hydrophilicity than the  
884 fabrics (Fig. 6). Evidently, electrospinning and following immersion in aqueous methanol  
885 induced structural rearrangements that exposed hydrophilic groups to the fibre surface.  
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889 HEMA grafting determined a decrease in the contact angle both in the fabrics and nanofibres;  
890 this result is not surprising, since polyHEMA possesses chemical functional groups (i.e. OH  
891 groups) which are expected to increase hydrophilicity. Analogous trends have been reported  
892 in the literature for HEMA-grafted silk fibroin films [25]. The decrease in the contact angle  
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901 values indicated that the HEMA-grafting results in the exposure of hydrophilic groups  
902 towards the fibre surface; these groups belong to polyHEMA and possibly also to the silk  
903 fibroin polypeptide chain, which was found to rearrange upon grafting. It cannot be excluded  
904 that these rearrangements expose hydrophilic residues to the fibre surface. Actually, the  
905 decrease in the contact angle observed going from the control silk fabric to the corresponding  
906 nanofibrous scaffold may be explained accordingly and is in agreement with the already  
907 reported trend of the Raman  $I_{850}/I_{830}$  tyrosine ratio, which revealed an increased exposure of  
908 this hydrophilic residue [26]. Unfortunately, no information on the tyrosine environment can  
909 be obtained for the grafted samples, due to the contribution of the polymeric component to  
910 this spectral range.  
911

912 Upon adding HBA in the grafting mixture, the contact angle was found to slightly increase  
913 with respect to the HEMA35-grafted samples. This trend may be explained according to the  
914 compositions of the samples as determined by Raman spectroscopy (data reported in Fig. 1  
915 (B)), by considering that in HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted samples,  
916 the HEMA content was lower than in the HEMA35-grafted samples and, at the same time, the  
917 HBA content increased. HBA monomer brings an OH group as in the case of HEMA, but in  
918 the former the aliphatic chain bringing this group is longer and thus has more hydrophobic  
919 properties. It is interesting to note that the contact angle value well correlated with the %  
920 HEMA weight gain; as shown in Fig. 6 (B), at decreasing HEMA content, increases in contact  
921 angle (and thus in hydrophobicity) occurred.  
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923 *Cytotoxicity tests allow the analysis of the *in vitro* viability of the cells in contact with the*  
924 *substrates. They are generally employed as preliminary tests to screen the ability of different*  
925 *substrates to affect cell behavior, as they are simple and have a high sensitivity. On the other*  
926 *hand, the ISO 10993-5 *in vitro* cytotoxicity test guideline [51] does not define one single*  
927 *standard test method but it describes testing schemes. The selection of the type of cytotoxicity*  
928 *test depends on the specific samples under analysis. Cytotoxicity tests are often performed for*  
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962 24-72 h when screening different substrates as in our case, to give evidence of the  
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964 biocompatibility of the materials in direct contact with cells.  
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966 Cell culture studies showed that after 3 days, the nanofibrous membranes behaved better than  
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968 the corresponding fabrics at the same culture time (Fig. 7). These trends are not surprising  
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970 since it is well known that the material surface chemistry and topography have a great  
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972 influence on cell adhesion and growth [52]. Pure silk fibroin substrates have been reported to  
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974 promote the growth of anchorage dependent mammalian cells, thanks to its hydrophilic  
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976 nature, as well as to its ability to interact with the negative charged surface of cell membrane  
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978 [1,53]. In general, moderately hydrophilic surfaces display a better affinity for cells than  
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980 hydrophobic ones and improvements in cell adhesion have been reported by grafting silk  
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982 fibroin with polyHEMA and even more with poly(acrylic acid) [25]; however, this parameter  
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984 is not the only one determining cell adhesion since very hydrophilic silk fibroin derivatives  
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986 may negatively affect cell binding [25], and the chemical nature of the groups exposed to the  
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988 surface of the substrate plays a crucial role as well as topography. On the other hand,  
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990 crystallinity has been reported to influence both hydrophilicity and surface roughness [54-56].  
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992 In fact, variations in crystallinity lead to changes in surface roughness on the nanometer  
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994 length scale, to which cells are extremely sensitive [54,55]. Moreover, an increase in the  
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996 surface crystallinity as a result of conformational changes in the amorphous domains into  $\beta$ -  
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998 sheet has been hypothesized to contribute to an increase in surface hydrophobicity [56].  
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1001 All these aspects must be taken into account to explain the data reported in Fig. 7. It is  
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1003 interesting to note that the conformational changes occurred in silk fabrics upon grafting with  
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1005 HEMA and HBA, as well as after electrospinning and treatment with aqueous methanol, did  
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1007 not prevent the natural cell recognition sites of the protein from being exposed towards the  
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1009 surface of the substrates; evidently, the surface chemistry and topography of the nanofibres  
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1011 appeared more favorable than those of the fabrics.  
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1015 In the future, we will perform *in vitro* cell experiments on nanofibrous samples for longer  
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1022 times in the perspective to screen suitable compositions for soft tissue regenerative medicine.  
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## 1026 **5. CONCLUSIONS**

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1028 In this study, *B. mori* silk fabrics were successfully modified by grafting with HEMA and a  
1029 binary mixture of HEMA and HBA. These substrates, as well as the corresponding  
1030 nanofibrous scaffolds obtained by electrospinning from TFA and treatment with aqueous  
1031 methanol, were characterized by different analytical techniques to gain information on their  
1032 morphological, chemical, physical and biocompatibility properties. Vibrational spectroscopy  
1033 was successfully used to gain information on the conformational rearrangements occurred in  
1034 silk fibroin upon grafting as well as on the composition of the samples, on which the trend of  
1035 the contact angle data were found to depend. PolyHEMA was found to hinder crystallization  
1036 into  $\beta$ -sheet only upon electrospinning and treatment with aqueous methanol; the presence of  
1037 the HBA component in the grafting mixture did not further decrease the ability of silk fibroin  
1038 to rearrange into  $\beta$ -sheet upon the same treatments, due to its low contents (below 5%) under  
1039 the used experimental conditions. However, at higher weight gains, the HBA component was  
1040 found to have a higher impact on silk fibroin conformation than the grafting with HEMA.  
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1056 Cell culture tests showed that the surface of the substrates under study was compatible with  
1057 fibroblasts attachment and growth. The HEMA25\_HBA10-grafted fabric induced a  
1058 significant improvement in cell proliferation with respect to control silk fabric; the  
1059 nanofibrous scaffolds under study showed a significantly higher cell growth than the fabrics,  
1060 suggesting that cell adhesion and proliferation may be easily modulated by varying the  
1061 surface chemistry and topography of the substrates. The results here presented demonstrate  
1062 that grafting improved the surface properties of silk fibroin for enhanced functional  
1063 performance in view of biomedical applications.  
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## CAPTIONS FOR FIGURES

**Figure 1.** (A) Raman spectra of HEMA35-grafted, HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted silk fabrics (weight gains of 26%, 24% and 20%, respectively).

The spectra of control silk fabric and polyHEMA [26] are reported for comparison. Some of the bands prevalently assignable to phenylalanine (F), tyrosine (Y) and tryptophan (W) are indicated. The bands assignable to polyHBA are indicated with a circle, those assignable to polyHEMA with an asterisk. The bands due to  $\beta$ -sheet ( $\beta$ ) and unordered (Un.) conformations are indicated. (B) % weight gains of HEMA and HBA in the HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted fabrics, as determined by using the  $I_{602}/I_{644}$  and  $I_{1300}/I_{644}$  intensity ratios calculated from the Raman spectra of Fig. 1(A), by interpolation from the lines reported in Fig. S4 (A) and (B), SM.

**Figure 2.** (A) Full-width at half maximum (FWHM) of the Amide I band, as obtained from the Raman spectra of the silk fabrics and electrospun nanofibres after immersion in aqueous methanol. The data corresponding to silk control samples [26] are reported for comparison.

(B) Percentages of secondary structure conformations as obtained by the curve fitting of the Raman Amide I range of control silk, HEMA35 and HEMA25\_HBA10 samples (fabrics and electrospun nanofibres after immersion in aqueous methanol). The data corresponding to the HEMA30\_HBA5 samples were not reported since they were not significantly different from those of the HEMA25\_HBA10 samples, due to the still lower HBA content.

**Figure 3.** (A) Raman spectra in the Amide I range of the HEMA-grafted and HBA-grafted fabrics (weight gains of 26% and 25%, respectively). (B) Percentages of secondary structure conformations as obtained by the curve fitting of the Raman Amide I range of the same samples.

**Figure 4.** Raman (A) and IR (B) spectra of electrospun HEMA35-grafted, HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted nanofibres after immersion in aqueous methanol. The spectra of control silk nanofibres treated under the same conditions [26] are reported for

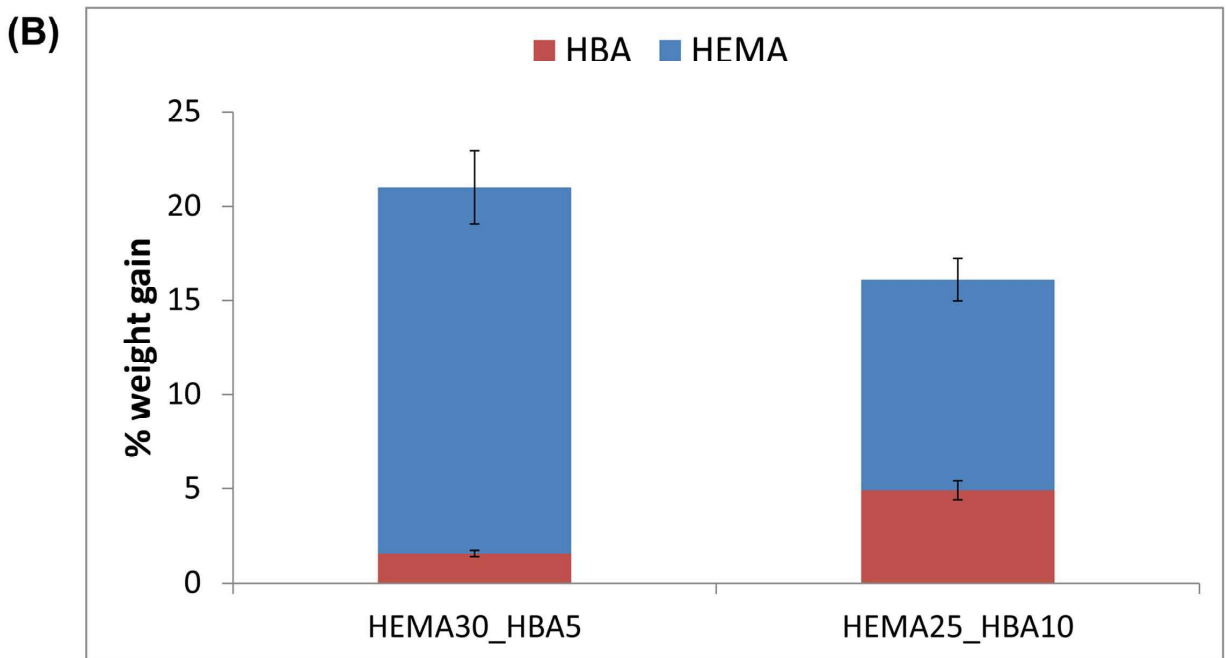
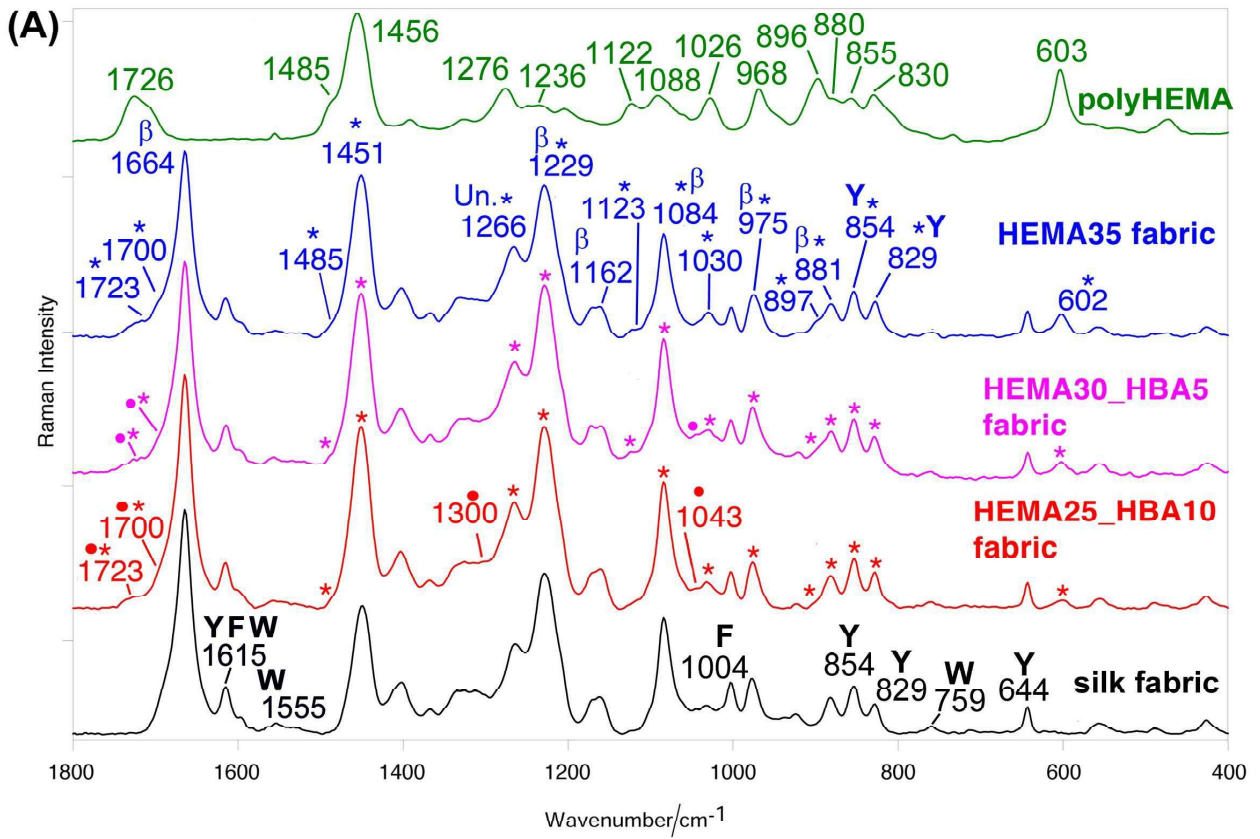
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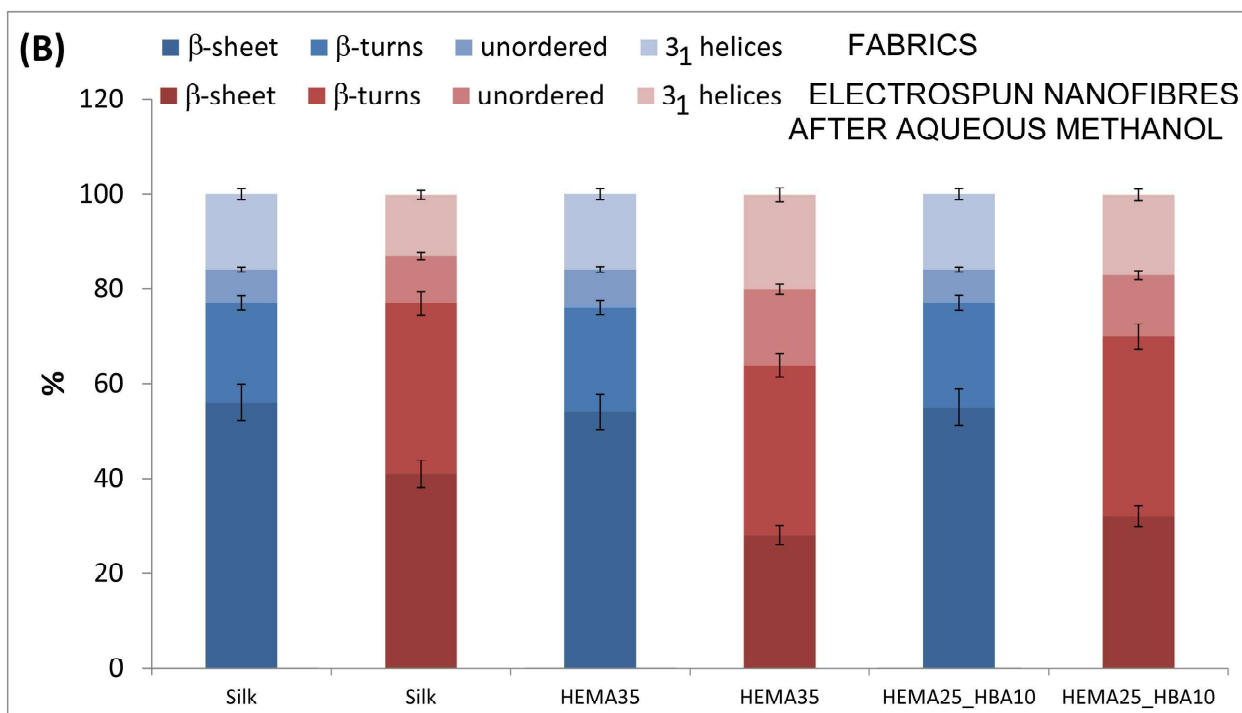
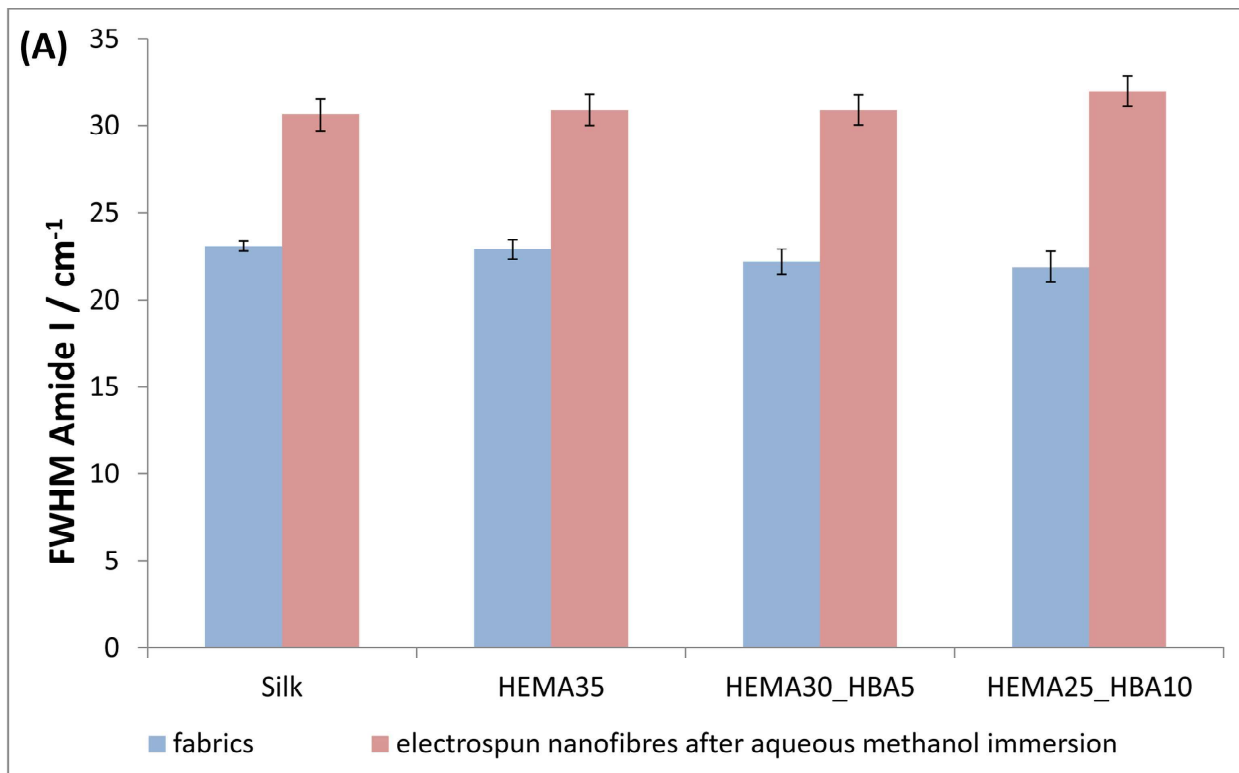
1504 The components having a contribution from aspartic acid (D) and glutamic acid (E) are  
1505 indicated. The bands assignable to polyHBA are marked with a circle, those assignable to  
1506 polyHEMA with an asterisk. The bands assignable to Silk I,  $\beta$ -sheet ( $\beta$ ) and unordered (Un.)  
1507 conformations are indicated.  
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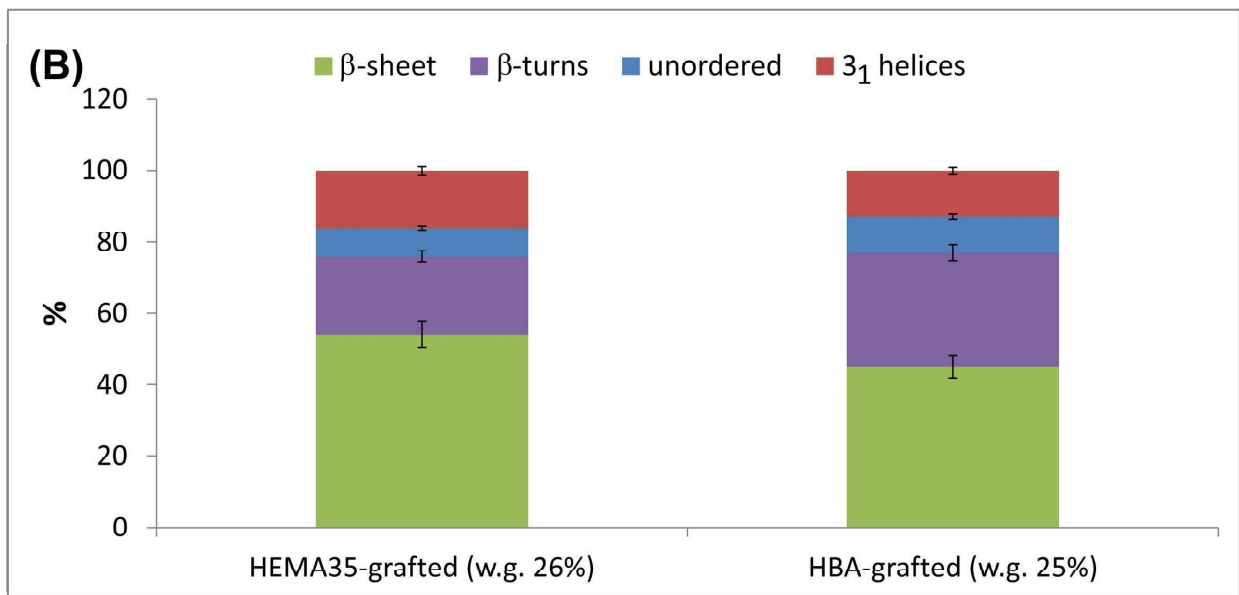
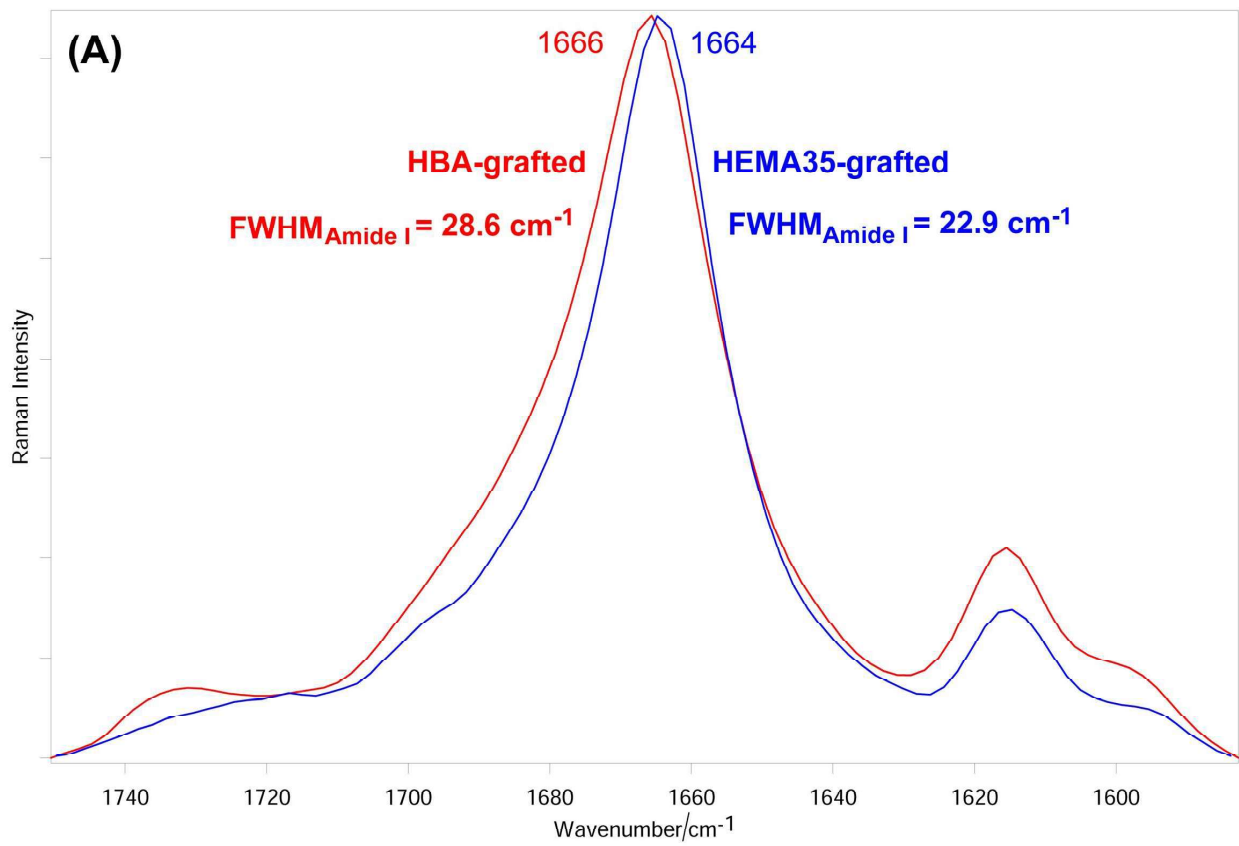
1512 **Figure 5.** SEM images of silk fabrics, as-electrospun nanofibres and nanofibres after  
1513 immersion in aqueous methanol referred to the following materials: untreated control silk ((a),  
1514 (b), (c)), HEMA35-grafted silk ((d), (e), (f)); HEMA30\_HBA5-grafted silk ((g), (h), (i));  
1515 HEMA25\_HBA10-grafted silk ((j), (k), (l)).  
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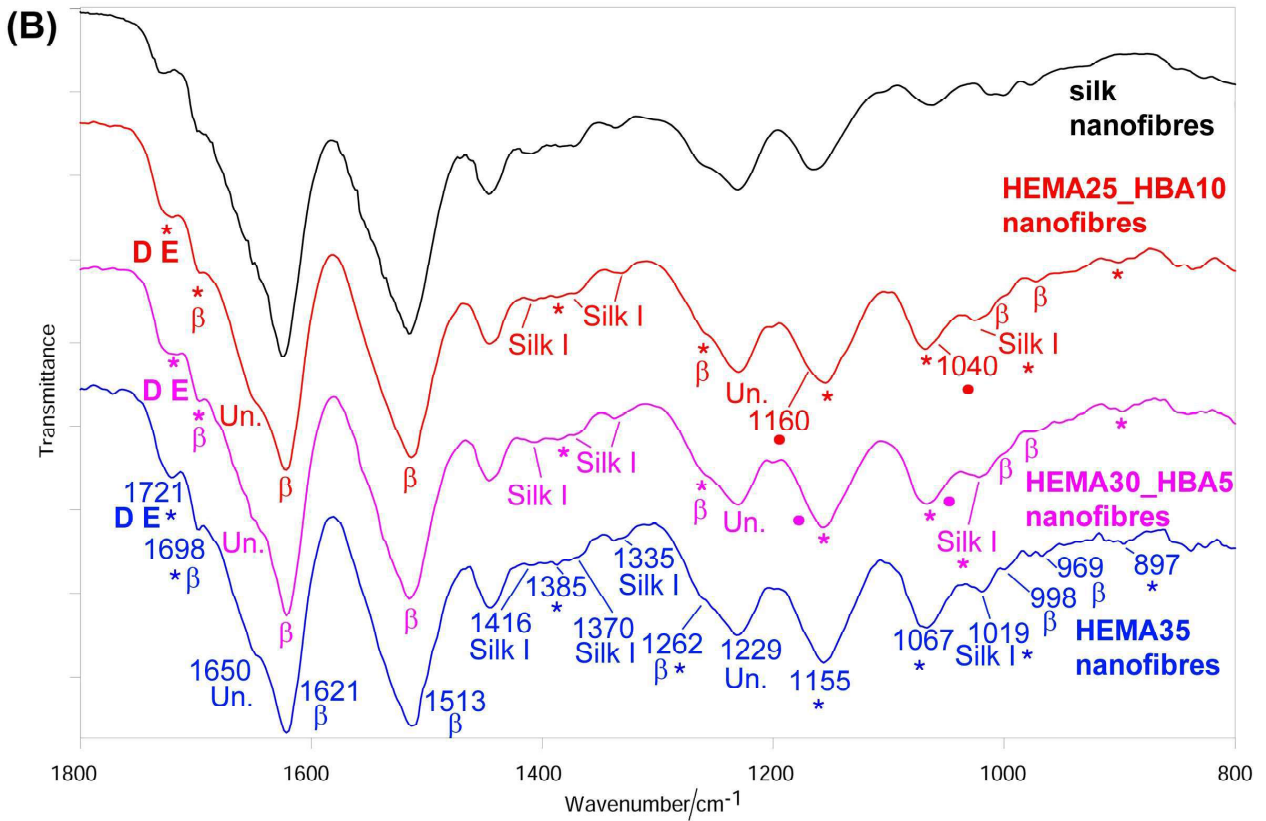
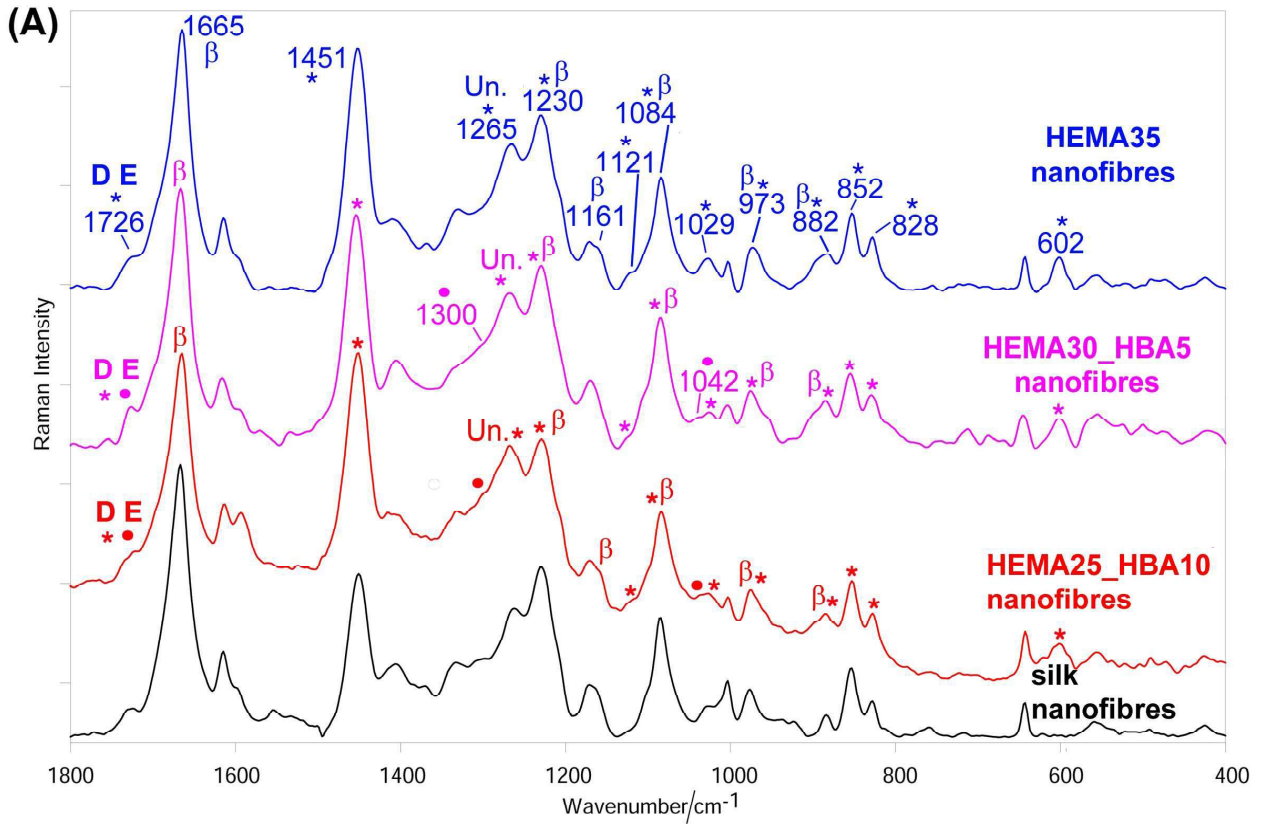
1521 **Figure 6.** (A) Contact angle values of the silk fabrics and nanofibres after immersion in  
1522 aqueous methanol. Data are average values and bars represent standard deviation. Asterisks  
1523 indicate statistically significant differences. (B) Trend of the contact angle values as a  
1524 function of the % HEMA weight gain (determined by weight measurements for HEMA35-  
1525 grafted samples and by Raman spectroscopy for HEMA30\_HBA5-grafted and  
1526 HEMA25\_HBA10-grafted samples, data reported in Fig. 1 (B)).  
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1534 **Figure 7.** Cellular viability determined by calcein-AM assay of NIH 3T3 fibroblasts exposed  
1535 for 1 and 3 days to the silk fabrics (A) and to the nanofibres treated with aqueous methanol  
1536 (B). Values labeled with same letters are not statistically different from each other, whereas  
1537 different letters indicate statistical differences ( $P < 0.01$ ). The asterisks indicate significant  
1538 differences between fabric and corresponding nanofibres, at each culture time.  
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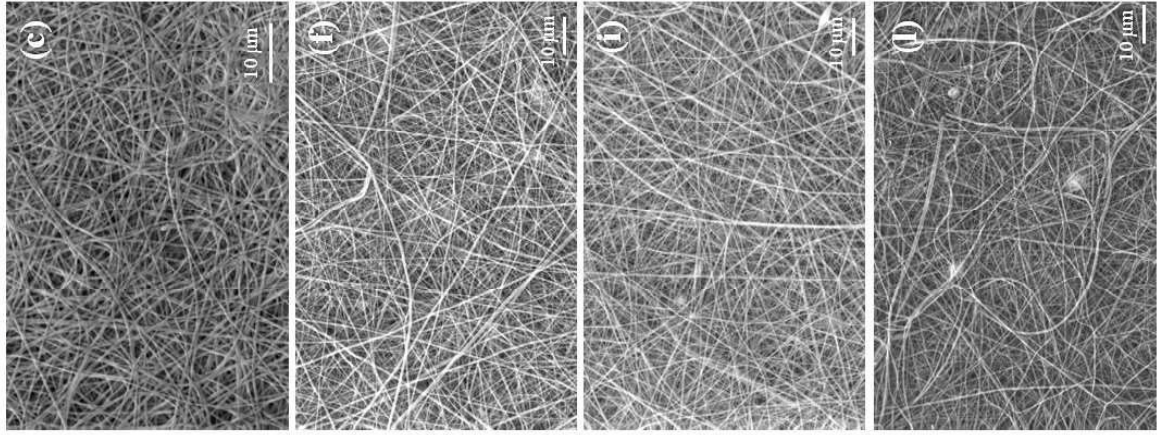




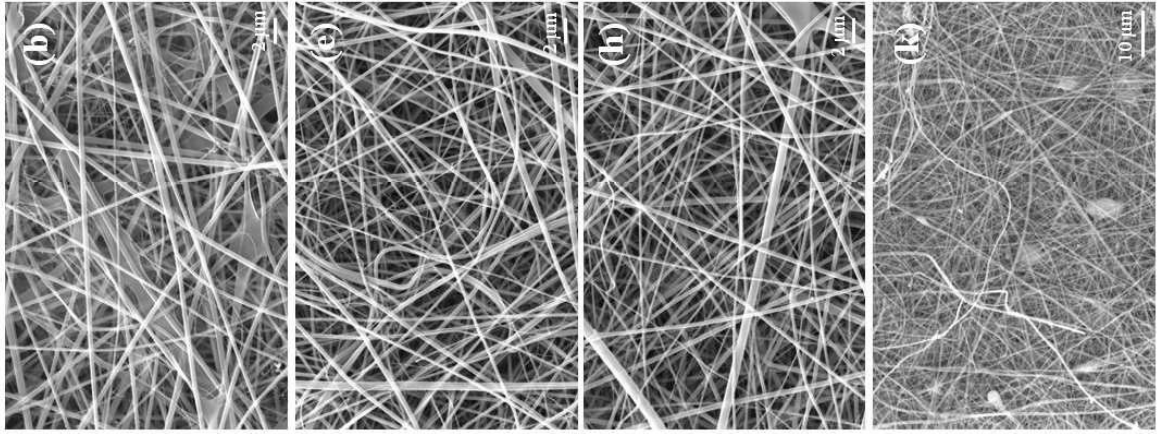




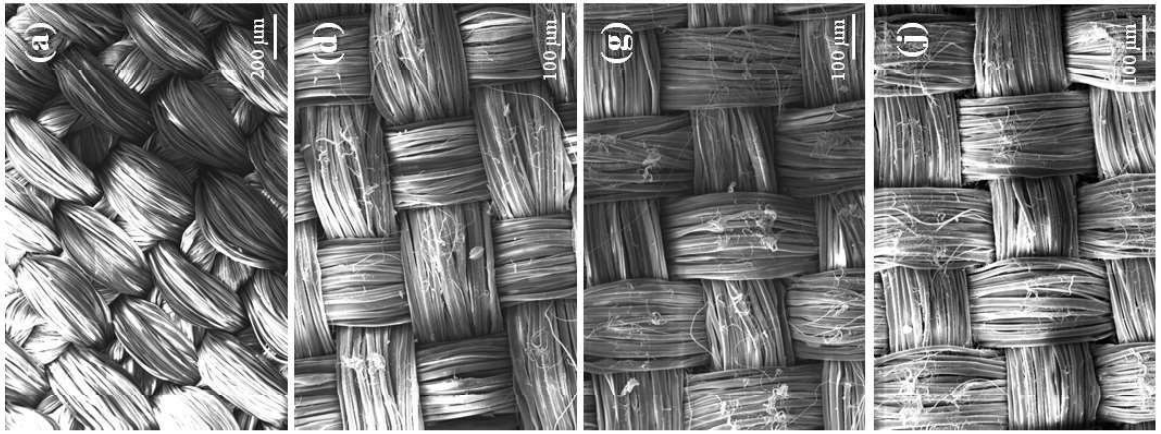
**Nanofibres after aqueous methanol**



**As-electrospun nanofibres**



**Silk fabrics**

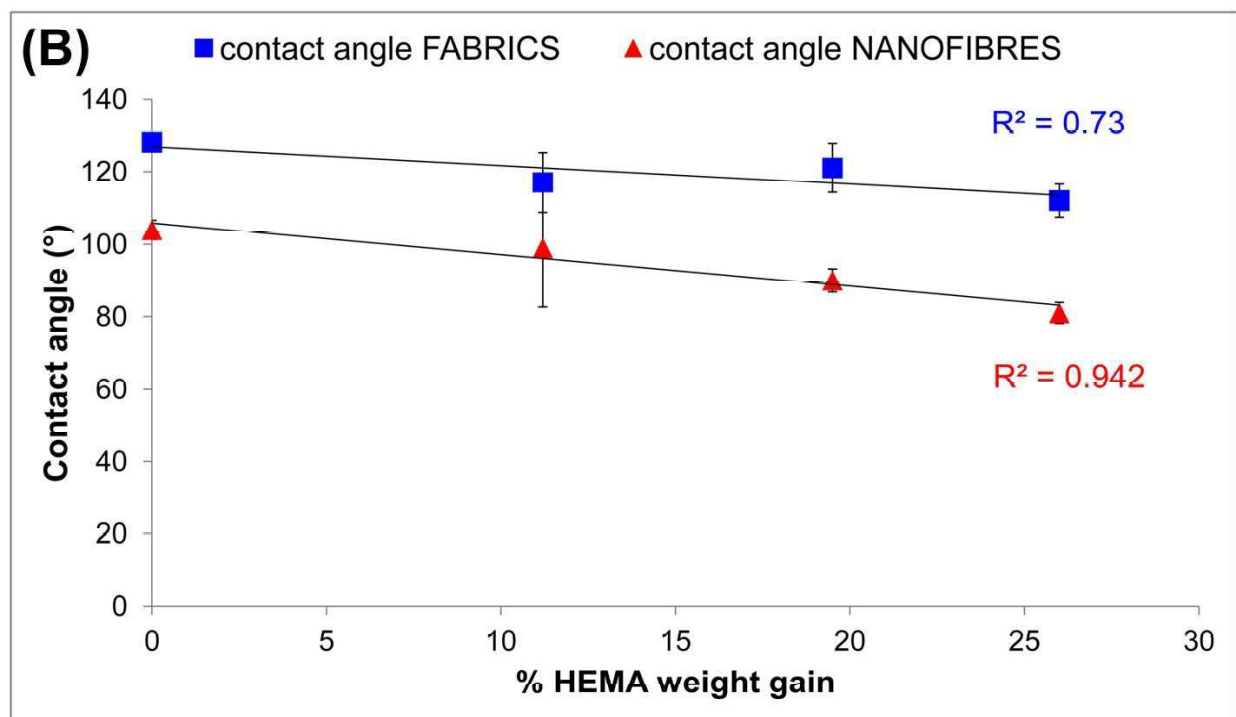
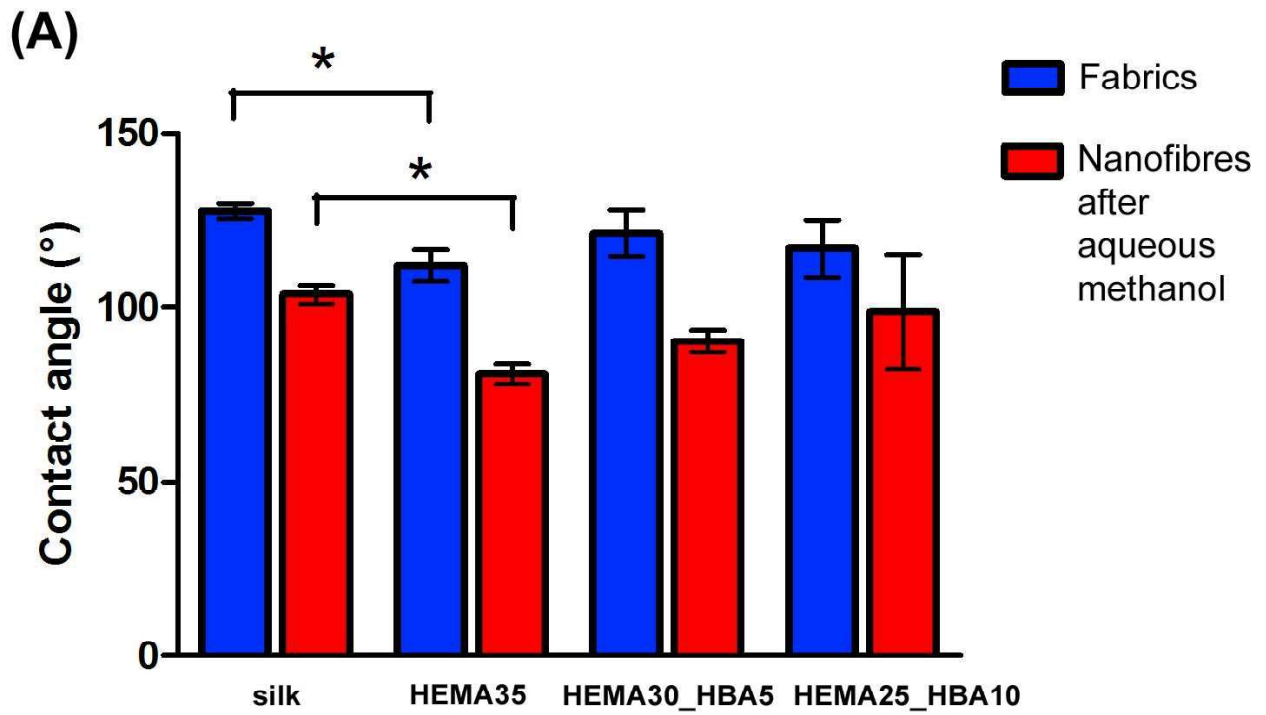


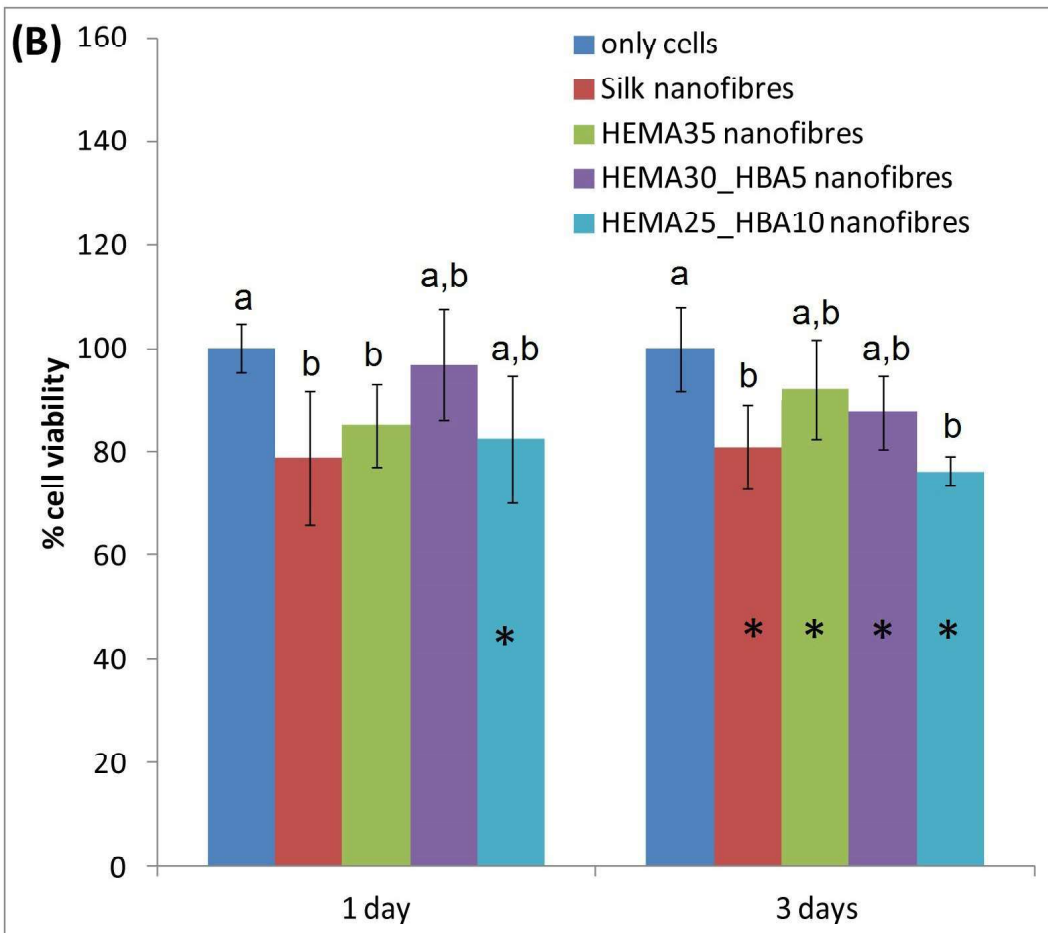
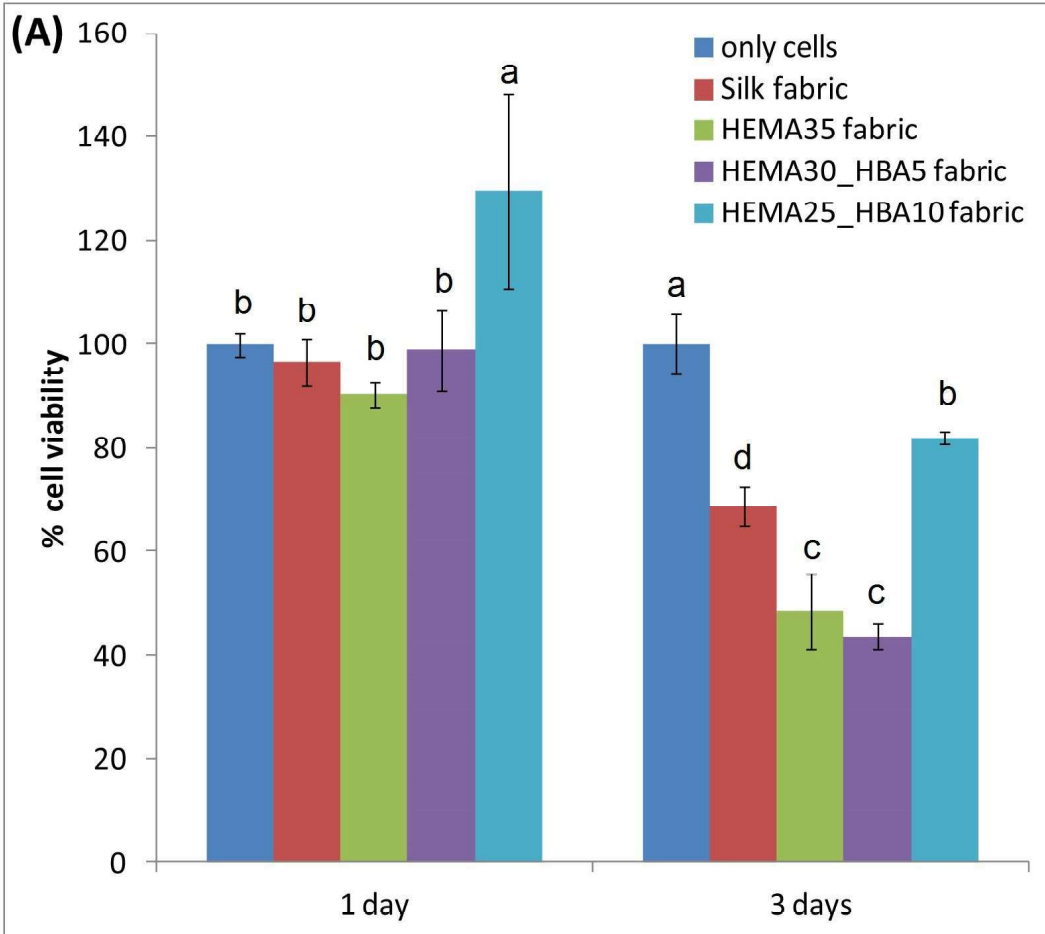
Untreated

HEMA35

HEMA30\_HBA5

HEMA25\_HBA10





***SUPPLEMENTARY MATERIAL***

**SILK FIBRES GRAFTED WITH 2-HYDROXYETHYL METHACRYLATE (HEMA) AND 4-HYDROXYBUTYL ACRYLATE (HBA) FOR BIOMEDICAL APPLICATIONS**

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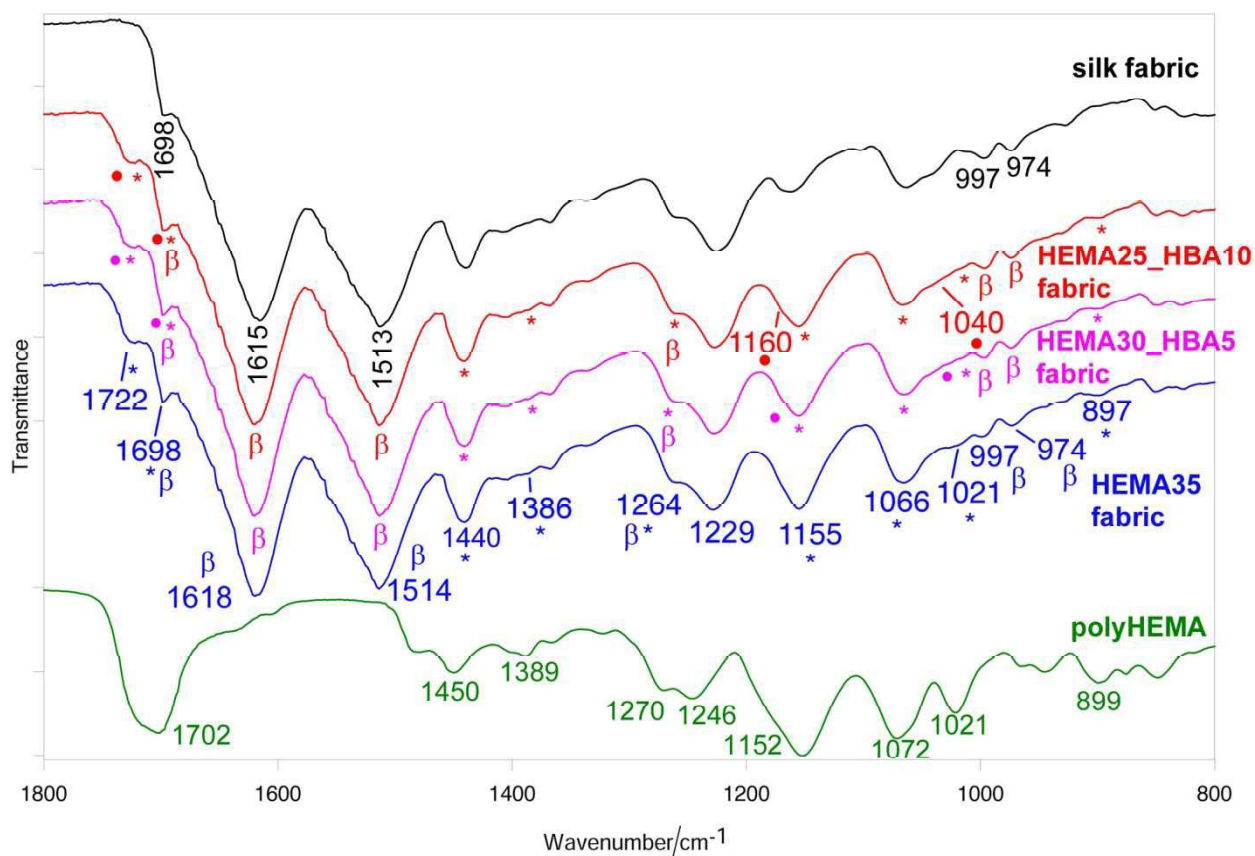
<sup>1</sup> Department of Biomedical and Neuromotor Sciences, University of Bologna, Via Belmeloro 8/2, 40126 Bologna, Italy

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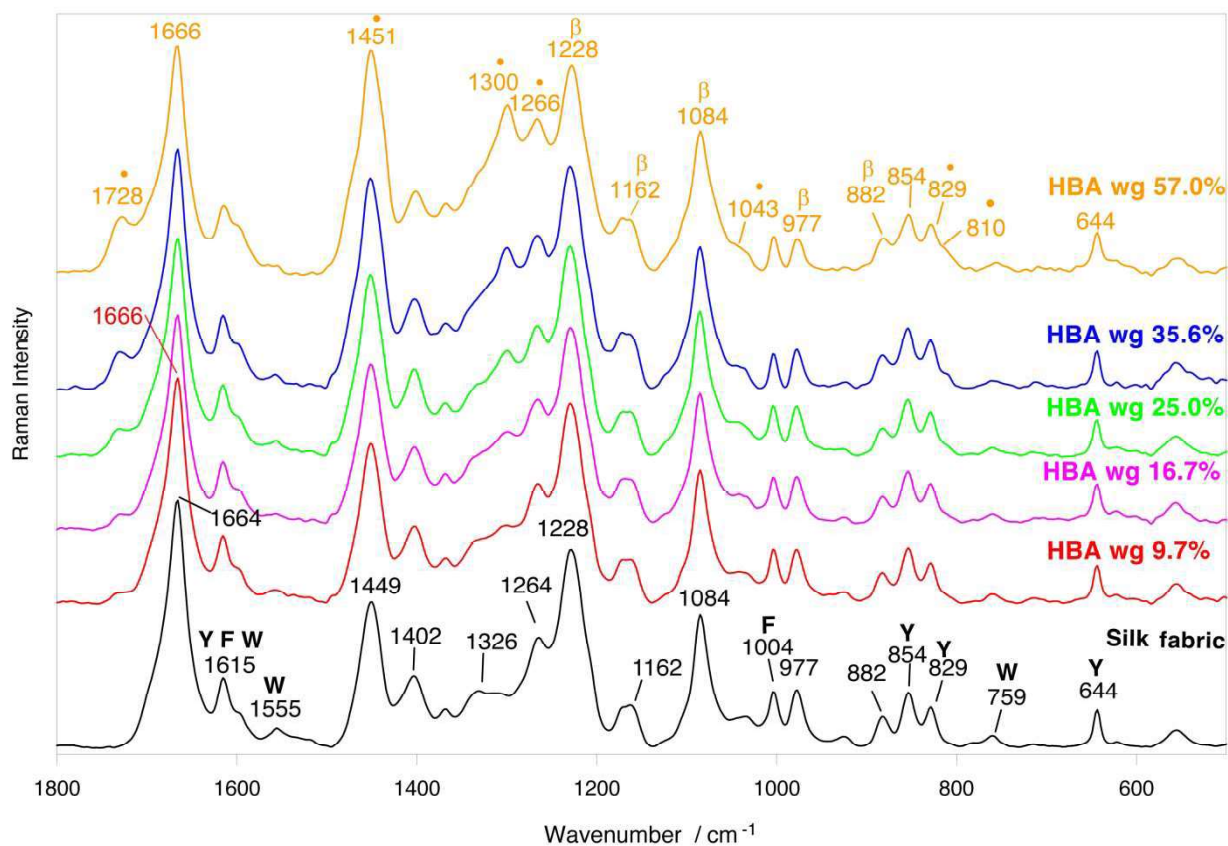
<sup>3</sup> Department of Sciences and Technological Innovation, University of Eastern Piedmont, Piazza Sant'Eusebio 5, 13100 Vercelli, Italy

<sup>4</sup> Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Turin, Italy

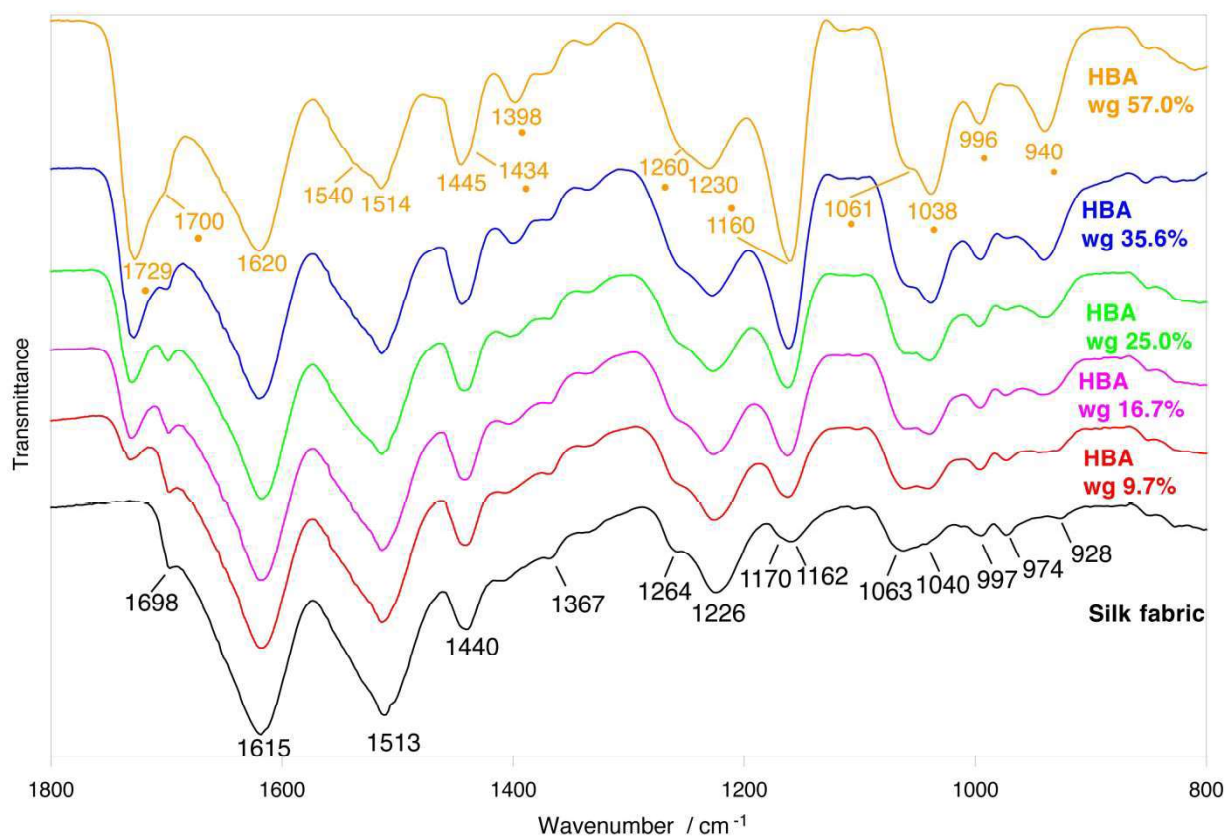
<sup>5</sup> Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano, Japan



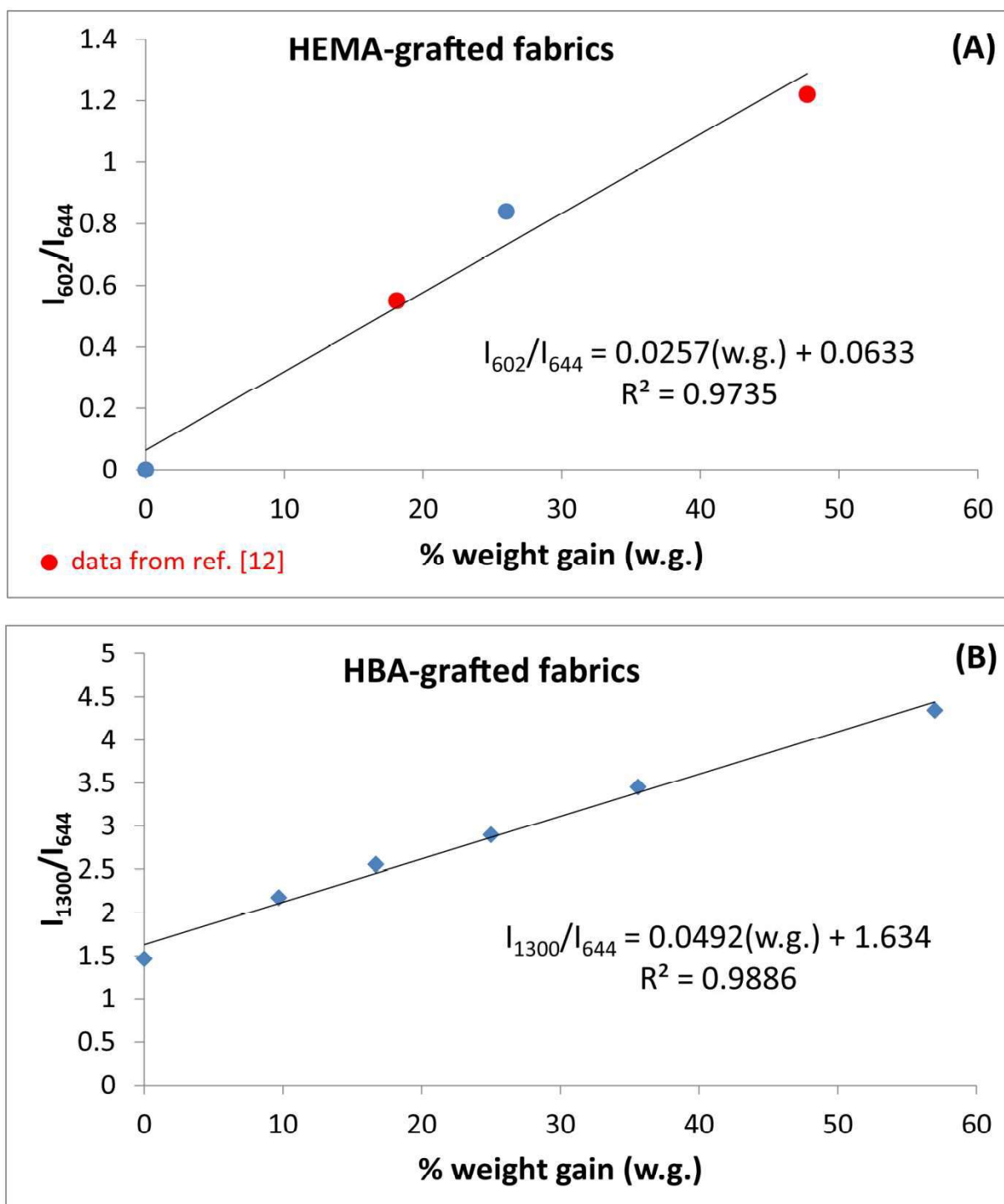
**Figure S1.** IR spectra of HEMA35-grafted, HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted silk fabrics (weight gains of 26%, 24% and 20%, respectively). The spectra of control silk fabric and polyHEMA [26] are reported for comparison. The bands assignable to polyHBA are indicated with a circle, those assignable to polyHEMA with an asterisk. The bands assignable to  $\beta$ -sheet ( $\beta$ ) conformation are indicated.



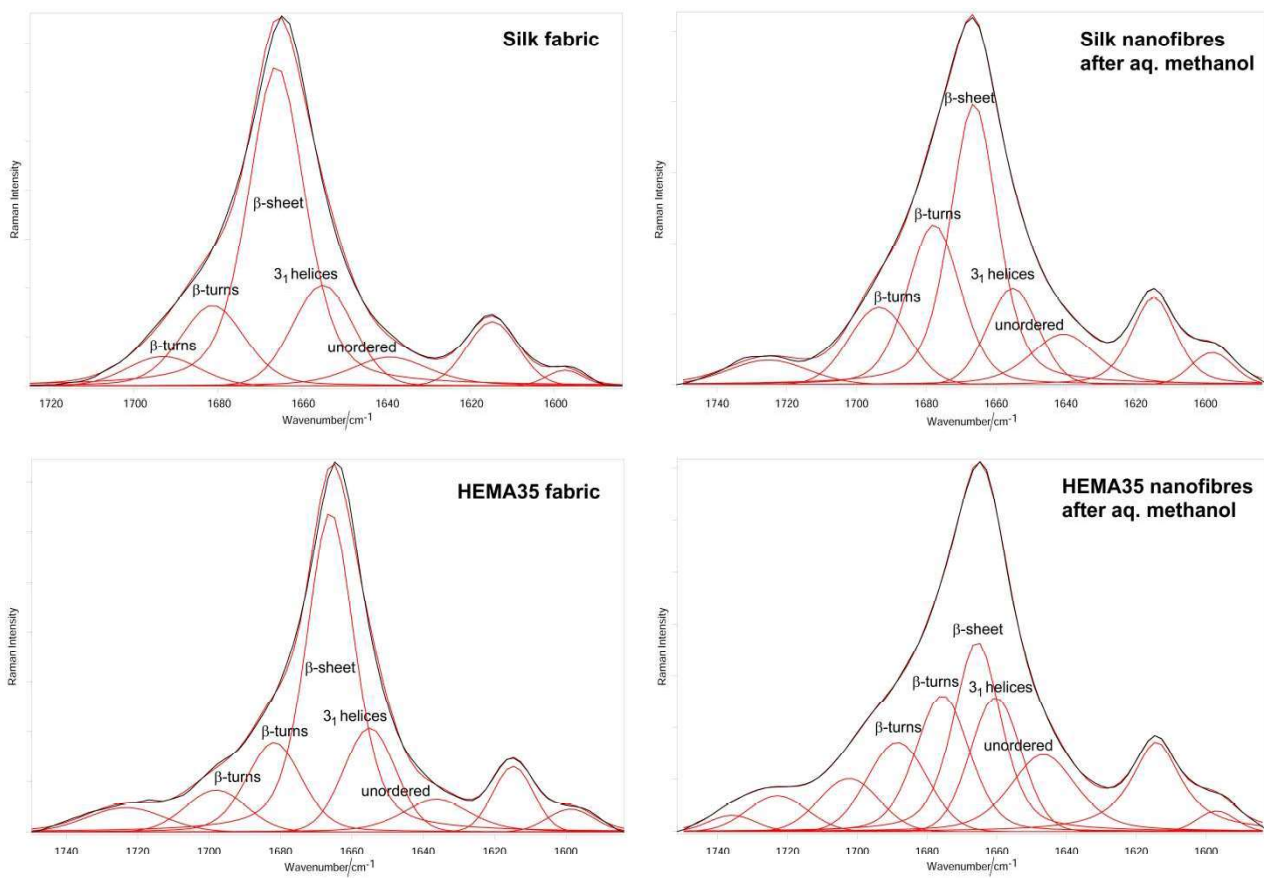
**Figure S2.** Raman spectra of the silk fabrics before and after grafting with HBA (weight gains of 9.7%, 16.7%, 25.0%, 35.6% and 57.0%). The spectra are normalized to the intensity of the Tyr band at 644  $\text{cm}^{-1}$ . Some of the bands prevalently assignable to phenylalanine (F), tyrosine (Y) and tryptophan (W) are indicated. The bands assignable to polyHBA are indicated with a circle. The bands due to  $\beta$ -sheet ( $\beta$ ) conformation are indicated.



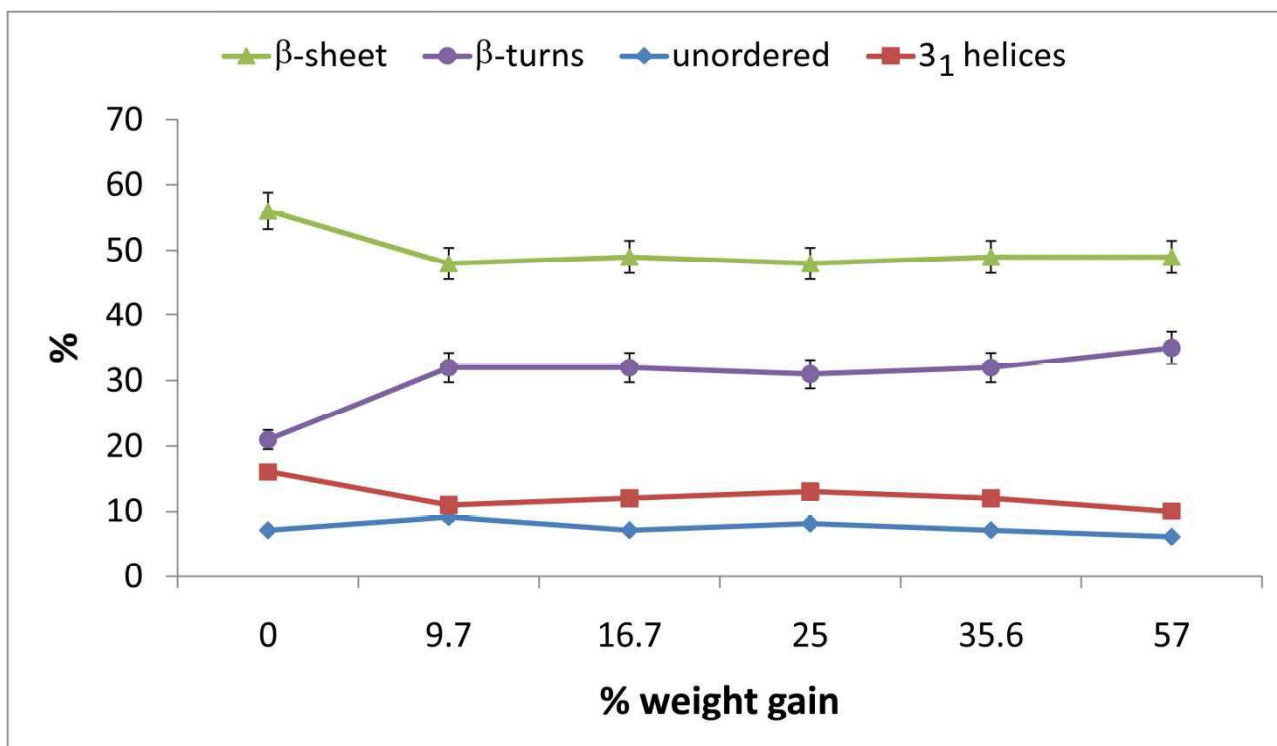
**Figure S3.** IR spectra of the silk fabrics before and after grafting with HBA (weight gains of 9.7%, 16.7%, 25.0%, 35.6% and 57.0%). The bands assignable to polyHBA are indicated with a circle.



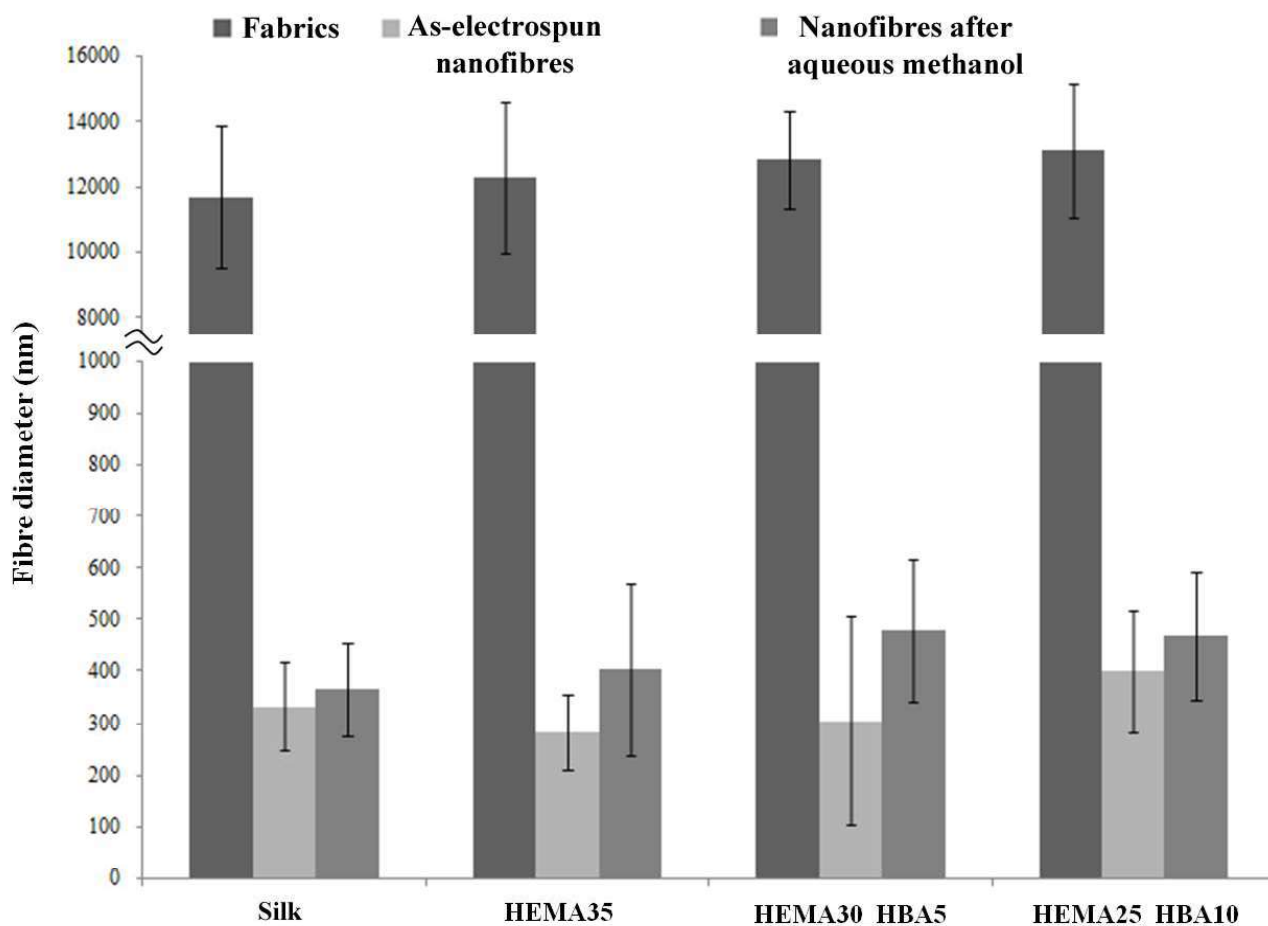
**Figure S4.** Trend of the Raman  $I_{602}/I_{644}$  (A) and  $I_{1300}/I_{644}$  (B) intensity ratios as a function of the % weight gain. The  $I_{602}/I_{644}$  values were calculated from the Raman spectrum of the HEMA35-grafted fabric (Fig. 1(A)) as well as from data reported in the literature on other HEMA-grafted samples (with weight gains of 18.1% and 47.7%) [12]. The  $I_{1300}/I_{644}$  values were calculated from the spectra reported in Fig. S2, Supplementary Material.



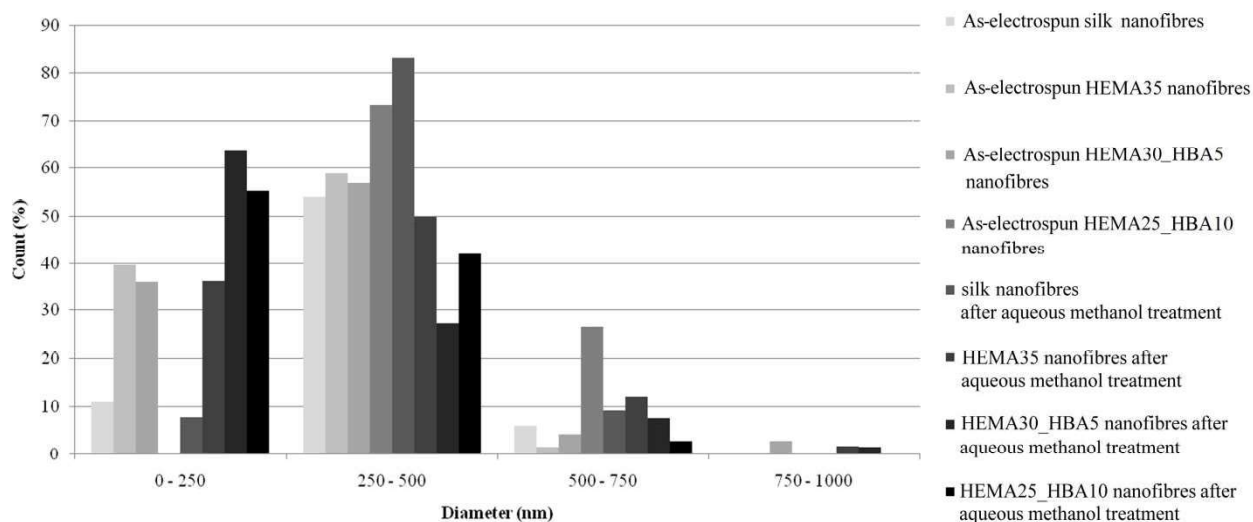
**Figure S5.** Experimental (black) and fitted (red) Raman Amide I region; the fitted components are shown.



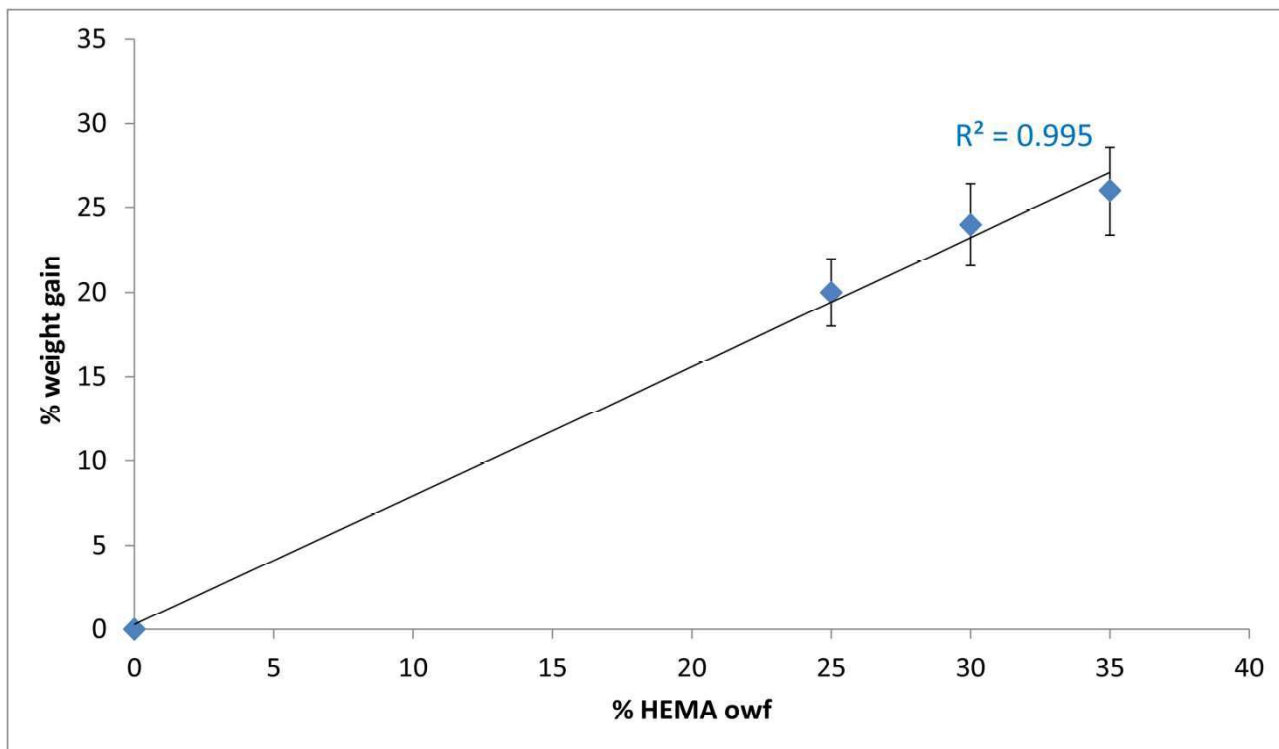
**Figure S6.** Trend of the secondary structures contents as a function of the % HBA weight gain, as obtained from the fitting of the Raman Amide I range of the HBA-grafted samples (Figure S2, Supplementary Material).



**Figure S7.** Fibre diameters of silk fabrics, as-electrospun nanofibres and nanofibres after immersion in aqueous methanol: control silk, HEMA35-grafted, HEMA30\_HBA5-grafted and HEMA25\_HBA10-grafted silk. Columns are the average values, bars represent the standard deviation.



**Figure S8.** Pore size distribution in as-electrospun control silk, HEMA35-grafted, HEMA25\_HBA10-grafted and HEMA30\_HBA10-grafted nanofibres after immersion in aqueous methanol.



**Figure S9.** Trend of the % weight gain of the grafted fabrics under study as a function of the % owf of HEMA in the grafting mixture.