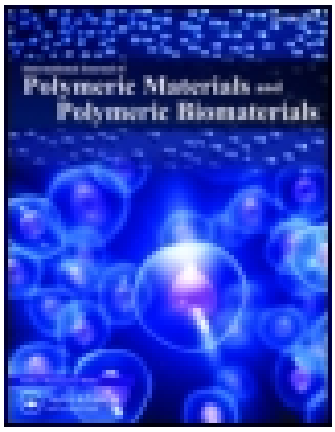

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Functional Gelatin-Carbon Nanotubes Nanohybrids with Enhanced Antibacterial Activity

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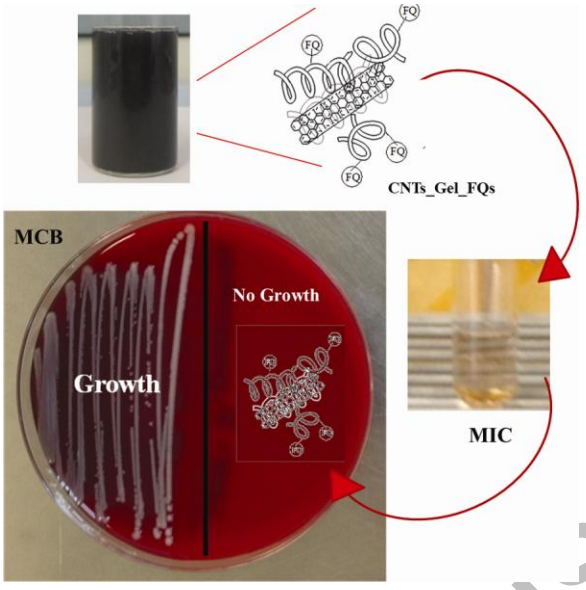
Abstract

Hybrid materials with enhanced antibacterial activity were prepared by incorporation of Carbon Nanotubes within gelatin-fluoroquinolones bioconjugates. Gelatin bioconjugates were characterized by UV-Vis, FT-IR, and calorimetric analyses, nanohybrids by morphological analyses. Biocompatibility was evaluated on Human Mesenchymal Stem Cells, and antibacterial performance against *Klebsiella Pneumoniae* and *Escherichia Coli*. Minimun Inibitory Concentrations from 0.025 to 0.05 μg mL^{-1} and from 0.025 to 0.10 μg mL^{-1}, and MBC from 0.025 to 0.10 μg mL^{-1} and from 0.05 to 0.20 μg mL^{-1} were detected for *Escherichia Coli* and *Klebsiella Pneumoniae*, respectively, showing that nanotubes increase antimicrobial activity comparing to both free and Gelatin-conjugated drugs.



KEYWORDS: Carbon Nanotubes, Gelatin Biocojugates, Fluoroquinolones, Antimicrobial Activity

INTRODUCTION

Diseases associated to infections by pathogenic microorganisms are one of the major causes of death among the mankind. Bacterial infections are of particular relevance in the case of biomedical devices and prosthesis, as well as in the hospital equipment [1–4] and thus several innovative biomaterials with remarkable antimicrobial properties have been proposed with the aim to reduce the healthy concerns associated to pathogens [5–7]. In this context, nanostructures including metal nanoparticles (e.g. silver nanoparticles) and carbon nanostructures have held a prominent place [3] Moreover, although possessing great efficiency, the use of metal nanoparticles is limited by their high permeation into surface and ground waters, with relevant pollution concerns [9–11], while carbon nanostructures show less environmental impact since they are less solved [12, 13]. Among the different carbon nanostructures, carbon nanotubes (CNTs), by virtue of their

structure conferring superior chemical, optical, electrical, thermal, mechanical and biological properties, are widely explored for the preparation of performing biomedical materials [14–16]. The whole of these properties, together with the favourable ability to strongly interact with different cell lines and easily undergo internalization processes [17], make these carbon nanostructures promising candidates for application in biomedicine, where CNTs-containing biomaterials are widely explored as novel and alternative diagnostics and therapeutics [18], as gene and drug delivery vectors [19], and, more recently, as emerging nanomaterials with strong antimicrobial efficiency [20, 21]. The antimicrobial activity of nanotubes has been attributed to the physical interaction between bacteria and CNTs [22, 23], which act as "nanosyringes" causing highly localized disruption of bacterial cell walls and membranes [24]. The detailed antimicrobial mechanism of CNTs, which was not observed at low concentrations [25], and found to strongly depend on CNTs diameter, length, concentration, aggregation state, and surface functionalization [26], involves a three-steps process: at first the contact between CNTs and bacteria occurs, then the bacterial membrane is perturbed and finally oxidized [27]. Although it was proved that pristine CNTs have higher antibacterial activity than functionalized CNTs, as a consequence of their tendency to form big aggregates better damaging the bacterial membrane [28], individual CNTs cannot be used within the human body, since they possess an asbestos fiber-like structure which is one the major concern in public health according to the definition of World Health Organization [29]. Needle particles, indeed, induce asbestos-like reactions causing foreign body mesothelioma [30]. Thus, CNTs surface must be firstly functionalized in order to overcome the intrinsic self-aggregation behaviour thus reducing the toxicity [24].

The most common approaches involves the non-covalent functionalization with suitable surfactants or macromolecules, and the covalent functionalization via oxidation or cyclo-addition reactions, followed by further reactions with suitable chemical species [31]. CNTs functionalization via non-covalent absorption of polymers has been reported as the simplest methodology to enhance their hydrophilic behaviour. Several different macromolecular systems has been proposed for this purpose [32], with the obtainment of hybrid materials widely applied in different technological fields, from engineering to electronics and biomedicine [33, 34].

In biomedicine, among the different polymeric materials explored to enhance the CNTs biocompatibility, protein materials, and gelatin in particular, were found to be very promising materials, since no toxicity on human healthy cells was recorded [35]. Furthermore, when CNTs are inserted into polymer therapeutics, the biological activity (e.g. anticancer, anti-inflammatory) is largely enhanced and the whole system is more effective than the separate components alone, with relevant potential application in the treatment of different diseases [36].

It should be considered that the enhanced water dispersibility of CNTs has a crucial effect on the reduction of antimicrobial activity, because the direct contact between CNTs and target pathogens is obstructed [22, 23, 37]. The ultimate mechanism of the CNTs antibacterial activity and toxicity is, indeed, based on the same CNTs properties, and thus the reduction of the toxicity on healthy human cells results is a reduction of the antimicrobial efficiency. As a consequence, great efforts have been devoted to improve

the CNTs antimicrobial activity by specific functionalization with antimicrobial molecule [38, 39], such as the antimicrobial peptide nisin [40], and the synthetic antibiotic cephalexin [41]. With respect to polymer therapeutics, an interesting approach is surely the use of antimicrobial macromolecular systems, a heterogeneous class of high molecular weight materials differing in both composition and mechanism of action [42]. Various antimicrobial systems have been proposed in literature [43], and recently we developed conjugates based on fluoroquinolones-type antibiotics (FQs) and gelatin (Gel) in order to match the biocompatibility of gelatin with the antibacterial activity of FQs, a well-known class of synthetic antibiotics widely used for the treatment of diseases associated to several bacterial infections [44]. The choice of Gel is related to its advantageous properties allowing the numerous applications of this natural polymer in pharmaceutics, food science, and biomedicine for the preparation of drug delivery devices, packaging materials, scaffolds, etc. [45]. The results showed the drugs moieties retain their antimicrobial activity after conjugation to Gel and the whole systems is able to inhibit the growth of *Escherichia Coli* and *Klebsiella Pneumoniae* in agreement with the data reported in European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI), the recorded Minimum Inhibitory Concentration (MIC) values are, indeed, similar to the un-conjugated parent drug.

Based on these considerations, in the present work we evaluate the possibility to enhance the antimicrobial activity of Gel_FQs bio-conjugates by insertion of CNTs. The obtained nano-composite materials are expected to be highly tolerated by healthy human cells and

able, at the same time, to efficiently interact with pathogenic bacteria by the activity of both FQs and CNTs. The introduction of superior antibacterial properties to hybrid materials composed of CNTs and Gel could greatly enhance the applicability of such materials, since the biocompatibility (Gel), mechanical strength (CNTs) and antibacterial activity are combined in a single composite, allowing the preparation of highly versatile materials.

FQs were conjugated to Gel by a straight-forward synthetic approach involving the radical grafting of FQs onto the protein side chains [44], while CNTs were subsequently inserted into the bioconjugates by an ultrasonication method [35]. The so obtained hybrid materials were extensively characterized by morphological analyses and in terms of biocompatibility on Human Mesenchymal Stem Cells (hMSCs). The antibacterial properties were tested on *Escherichia Coli* and *Klebsiella Pneumoniae* and compared to the un-conjugated FQs and the Gel_FQs bio-conjugates to highlight the effect of each component on the recorded activity.

EXPERIMENTAL

Synthesis And Characterization Of Gel_Fqs Conjugate

The conjugates were synthesized as follows according to the optimized conditions reported in literature [44]. Briefly, in a 50 mL glass flask, 0.50 g of gelatin (PhEur, Bloom 160) were dissolved in 47 mL of H_2O, then 3.0 mL H_2O_2 3.0 M (9.0 mmol) and 0.20 g of ascorbic acid (1.1 mmol) were added and the mixture was maintained at 25°C under an inert atmospheric (all chemicals from Sigma

Chemical Co., St. Louis, MO). After 2 h, 0.1 mmol of FQs (Ciprofloxacin - CP, Levofloxacin - LV, and Lomefloxacin - LM) were added to solution and, after 24 h, the solution was introduced into dialysis tubes (6-27/32", MWCO: 12-14000 Da, Medicell International LTD) and dipped into a glass vessel containing distilled water at 20°C for 48 h with six changes of water. The resulting solutions were frozen and dried with a freeze dryer (Micro Modulyo, Edwards) to afford vaporous solids, coded Gel_CP, Gel_LV, and Gel_LM. Blank gelatin (Gel_B), acting as a control, was prepared when the reaction was carried out in the absence of FQs. The conjugates were checked to be free of un-bonded FQs analyzing the dialyses media by high-pressure liquid chromatography (HPLC) equipment made up by a Jasco PU-2089 Plus liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 µL loop), a Jasco UV-2075 HPLC detector (operating at 280 nm for CP and LM, at 294 nm for LV) and Jasco-Borwin integrator. A reversed-phase C18 column (µBondapak, 10 µm of 250 × 4.6 mm internal diameter obtained from Waters) was used. As reported in literature, the mobile phases for FQ (flow rate of 1.0 mL min^{-1} and injection volume of 20 µL) were as follows (all solvents from Carlo Erba, Milan, Italy): CP - mixture of 2% acetic acid aqueous solution and acetonitrile (84:16, v/v) [46]; LM - mixture of 1% acetic acid aqueous solution, acetonitrile and methanol (70:15:15, v/v/v) [47]; LV - mixture of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v) [48].

The Gel_FQs conjugates were characterized by means of FT-IR (KBr pellets on a Jasco FT-IR 4200, Jasco Europe s.r.l., Milan, Italy) and UV-Vis spectrometry

(Perkin Elmer Lambda 900 spectrophotometer for the absorption spectra; Perkin Elmer LS 50B spectrofluorimeter, equipped with Hamamatsu R928 photomultiplier tube for the corrected emission spectra, all confirmed by excitation ones) to determine the effectiveness of the derivatization process and the functionalization degree expressed as FD% according to the equation 1.

$$FD(\%) = \frac{\text{FQs (g)}}{\text{Gel\_FQs (g)}} \times 100 \qquad (1)$$

Calorimetric analysis of the samples was carried out using a Netzsch DSC200 PC. In a standard procedure about 6.0 mg dried sample were placed inside a hermetic aluminum pan, and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 80 to 350°C under a dry nitrogen atmosphere with a flow rate of 25 mL min^{-1} and heating rate of 5°C min^{-1}.

The size and distribution of CNTs_Gel_FQs were determined by dynamic light scattering (DLS) analysis using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corp, USA) at 25.0 ± 0.1 °C by measuring the autocorrelation function at 90°. The laser was operating at 658 nm. The distribution size was directly obtained from the instrument fitting data by the inverse "Laplace transformation" method and by Contin [49]. The polydispersity index (PI) was used as a measure of the size distribution. All the samples (1 mg mL^{-1} in distilled water) were analysed at different time intervals after the preparation (0.5, 24, 38, and 72 h). Each sample was measured three times, and results are expressed as means ± the standard deviation (SD).

Synthesis Of Cnts

Multi-walled CNTs were synthesized applying a so-called aerosol assisted chemical vapor deposition method as described in literature [50], using ferrocene as metal organic catalyst precursor and cyclohexane as carbon source. An excitation frequency of 850 kHz and a carrier gas flow consisting of 100 sccm Ar were used. The as-grown material was subsequently purified in order to eliminate the amorphous carbon and the catalyst particles by using a two-step method including a thermal treatment at 450°C in air for 1 h and an acid treatment using hydrochloric acid. The morphology of the samples was analyzed by utilizing a Scanning Electron Microscope (NOVA NanoSEM 200 [0-30 kV], Comm. FEI Company, Hillsboro, OR, USA) and a Transmission Electron Microscope (HRTEM/Tecnai F30 [300 kV] FEI company). Therefore, samples were grounded in an agate mortar and deposited onto self-adhesive, conducting carbon tape (Plano GmbH, Wetzlar, Germany) for SEM analysis. TEM samples were prepared by pressing the powdery CNT-composite between two small slides of aluminium foil on a Cu TEM grid (200 mesh, Plano GmbH, Wetzlar, Germany).

Preparation And Characterization Of Cnts_Gel_Fqs Composites

The non-covalent hybrid materials were synthesized by dispersing selected amount of CNTs into a fixed amount of Gel_FQs conjugates to reach the final ratio of 1% (w/w) using sonication by means of a cup-horn high intensity ultrasonic homogenizer (SONOPULS) with a cylindrical tip. The sonicator was operated at amplitude of 70% of the maximum and the duration of sonication was 30 min [35].

Biocompatibility Assay

Human Mesenchymal Stem Cells (hMSCs, Lonza; Walkersville MD-USA) were cultured in DMEM supplemented with 10% of pooled human AB type serum (PhABS, Lonza; Walkersville MD-USA) as previously reported [51]. Cells were maintained at 37°C in a saturated-humidity atmosphere of 95% air and 5% CO_2. In order to test the effect of CNTs_Gel_FQs, we prepared modified culture medium by mixing nanocomposite solutions with the complete culture medium. We obtained six modified culture media with different CNTs concentration: 5.0 μg mL^{-1}, 10.0 μg mL^{-1}, 15.0 μg mL^{-1}, 20.0 μg mL^{-1}, and 25.0 μg mL^{-1}. The complete culture medium was used as control sample. WST-8-(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4 disulfophenyl)-2H-tetrazolium mono-sodium salt), cell proliferation assay (Sigma Chemical Co., St. Louis, MO) was used to investigate the effect of the nanocomposites on cell viability. 25×10^3 cells were seeded into each well of a 96-well plate and then incubated with the different culture media. After 24 h incubation with nanocomposites at different concentrations, we added 10 μl of WST-8 solution in each sample and we incubated cells again for 2 h. The mitochondrial dehydrogenase of viable cells reduces the water-soluble WST-8 tetrazolium salt. The absorbance of test and control assays was performed using a Versamax microplate reader (Molecular Devices) at a wavelength of 450 nm with the reference wavelength 650 nm. The results are expressed as percentage of the control assays. Each experiment was carried out in quintuplicate; data were expressed as means (±SD).

Evaluation Of The Antibacterial Activity

The antibacterial activity of free FQs, Gel_FQs and CNTs_Gel_FQs hybrid materials was determined by the agar dilution method. *Escherichia coli PTCC2433* and *Klebsiella pneumonia PTCC4231* were grown in tryptic soy broth at 37°C overnight to obtain a 0.5 Mac Farland turbidity value. To assess the antibacterial activity, a series of fourteen tubes containing 0.5 ml of sterile culture medium (Mueller-Hinton broth, Biomerieux, Marcy-l'Étoile, France) was prepared. Considering the derivatization degree, an aliquot of composite solution was added to the first tube in order to raise a FQs equivalent concentration of 100 µg mL^{-1}. 0.5 mL of this solution was added in the second test tube, mixed and then moved to the third tube. The procedure was repeated for all the test tubes (removing 0.5 mL of solution from the last one) up to reach a FQs equivalent concentration of 0.025 µg mL^{-1}. Subsequently, in each tube, 30 µL of bacteria cultures were inoculated and left to incubate at 37°C for 24 h. After the incubation time, the turbidity of the tubes was evaluated and the Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration able to completely inhibit the bacterial growth.

To determine the Minimal Bactericidal Concentration (MBC), 0.1 mL from each broth tube showing no turbidity was inoculated onto agar plates. The MBC was defined as the lowest concentration of antibiotic at which no colonies grew on the plates after 24 h of incubation at 37°C. Each experiment was carried out in quintuplicate; data were expressed as means (± SD).

RESULTS AND DISCUSSION

Synthesis And Characterization Of Cnts And Gel_Fqs Bioconjugates

The first step for the preparation of antibacterial hybrid materials was the synthesis of the two components: CNTs and Gel_FQs bioconjugates.

CNTs were prepared by a Chemical Vapour Deposition method because this synthetic strategy allows a precise control on the CNTs shape and purity. The obtained carbon nanostructures were purified by thermal and acidic treatments for removing the catalyst particles and the amorphous carbon from the CNTs outer surface [34]. By a combined SEM-TEM analysis (Figure 1), it was determined that pristine CNTs exist as individual filaments made up of 20-30 graphene walls with a length of 5-15 μm, outer diameters of 10-80 nm, and inner diameters of 5-25 nm. They show few defects on the outer surface and are totally unable to be dispersed in water due to the formation of big agglomerates.

By ICP-MS and ICP-OES, in the as-grown state the Fe-content was found to amount between 2 and 7 wt % [34]. The Gel_FQs bioconjugates were prepared according to the literature data [44]. By virtue of its water compatibility and environmental safe behavior, the hydrogen peroxide/ascorbic acid redox initiated radical grafting of FQs onto the Gel was selected as synthetic strategy to promote the covalent insertion of Ciprofloxacin (CP), Levofloxacin (LV), and Lomefloxacin (LM) onto the protein side chain. The reaction, indeed, operates at room temperature in a totally aqueous media, without the formation of toxic side-

products [36]. In our previous work, we optimized the reaction conditions in terms of molar ratio between Gel and FQs. The reaction mechanism involves the reaction between the macro-radicals formed onto the protein structures with the aromatic ring of the quinolone moiety, probably by replacing the fluorine atoms, or the hydrogens atom and the piperazine ring in ortho- positions with respect to the halogen (positions 5, 6 and 7), without effecting the essential positions for the antimicrobial activity (positions 2, 3, and 4) (Figure 2) [44].

According to the literature, the presence of the FQs moieties inside the protein network was assessed by FT-IR measurements, allowing to the detect the FQs signals (Figure 3A); while the bathocromic shift of the UV-Vis absorption and emission peaks related to the aromatic portions of the FQs moieties inside the conjugate was employed as a confirmation of the covalent conjugation (Figure 3B).

The grafting reaction was performed by the optimized experimental conditions (0.1 mmol of FQs per grams of protein) to obtain the higher derivatization degree, expressed as mg of FQs equivalents for g of conjugates, as assessed by UV-Vis analyses. The recorded FD% values were 0.04 for Gel_CP, 0.09 for Gel_LM and 0.36 for GL-LV. As explained in the previous study, the different FD% was related to the different ability of the FQs to undergo grafting reaction, the differences in the chemical structures, indeed, allow the formation of different radical species with different reactivity.

Preparation And Characterization Of Cnts_Gel_Fqs Hybrid Materials

As before reported, although possessing superior chemical, physical and mechanical properties, the use of CNTs in biomedical field is obstructed by toxicity concerns [50]. In a previous work, we showed the possibility to greatly enhance the CNTs biocompatibility by embedding into a protein structure [35], specifically we set a sonication methodology of CNTs into a gelatin solution to produce hybrid materials (CNTs_Gel) with enhanced water dispersibility upon time.

The stability of the CNTs-Gel_FQs dispersion were extensively characterized by Dynamic Light Scattering and the results related to a composite dispersion of 1 mg mL^{-1} show an average particle size of 986 nm with a Polydispersivity Index of 0.005. The same data were obtained for all composites, since the dispersivity is a function of the Gel content. Although it is difficult to evaluate the particle size of anisotropic CNTs using DLS because CNTs are not spherical compounds, the absence of big aggregate in the micrometre range indicate the good dispersivity [51], which is retained over time, since no significant changes in the instrumental results have been observed over time (from 0.5 to 72 h).

The nanohybrids are characterized by a totally bio-compatible behavior on hMSC, which were selected as an in vitro model to assess the biocompatibility of chemicals and biomaterials since their metabolic pathways confer them high susceptibility to foreign molecules [52]. The results show that the cell viability was

no significantly affected by the CNTs_Gel composite materials in all the tested concentrations.

With the aim to test the antimicrobial activity, we used two gram-negative bacteria of Enterobacteriaceae family, *Escherichia Coli* and *Klebsiella Pneumoniae*, as pathogens, since they are responsible for several serious pathological infections, such as urinary, hematological, intra-abdominal, respiratory, cerebrospinal infection, and sepsis [44]. When considering the activity of CNTs_Gel composite materials, it was observed that the antimicrobial activity is totally suppressed after functionalization, thus with the aim to prepare effective antimicrobial hybrid materials, we selected the Gel_FQs conjugates as coating elements for the CNTs outer surface (Figure 4).

The composites were prepared according to the optimized sonication method [35], by employing a ratio of CNTs to Gel_FQs of 1/100 w/w), to obtain a well dispersed suspension, which was found to be stable over time, since no signs of deposition were observed even after 1 month (Figure 4). The morphology of the samples after functionalization was assessed by SEM and depicted in figure 1 (panels C, D) which reports the micrographs for pristine and functionalized CNTs. In the latter case, CNTs form a dense and robust network structure totally embedded within the polymeric matrix, and they appear as individual filaments emerging from the protein agglomerates.

To test the performance of the hybrid materials, comparative experiments were performed on FQs, Gel_FQs and CNTs_Gel_FQs, by considering the derivatization degree of each material. The sensitivity of microbial species can be categorized as susceptible or resistant according to the EUCAST for CP and LV, and CLSI for LM [53, 54]. Considering this classification, a pathogen is defined susceptible and resistant to CP when the MIC are 0.5 and 1.0 μg mL^{-1}, respectively; susceptible and resistant to LV for MIC of 1.0 and 2.0 μg mL^{-1}; while values of 0.5 and 2.0 μg mL^{-1} are an indication of susceptibility and resistance to LM.

In separate experiments, suitable amounts of each sample were dissolved in sterile test tubes containing the culture media, to reach final concentrations in the range 100 - 0.012 μg mL^{-1}. Figure 5 reports some of the test tubes, which were incubated with the bacteria to assess the performance of the samples as MIC, expressed as FQs equivalents.

It was observed that the linkage to the protein did not affect the CP antibacterial activity, since the MIC values were unmodified. The conjugates containing LV and LM retain a significant antibacterial activity, even if lower when compared to the corresponding free drugs. The antibacterial activity was in the order: Gel_CP > Gel_LM > Gel_LV for both the bacteria (Table 1).

When considering the tests performed in the presence of the CNTs_Gel_FQs composites, a totally different behavior is detected. In CP and LV cases, a

significant increase of the antibacterial activity is detected, and the MIC values decreases accordingly. Specifically, the presence of CNTs inside the CNTs_Gel_CP composite increases the CP activity in comparison to both the Gel_CP conjugate and the free CP by two and four times for *E. Coli* and *K. Pneumoniae*, and the MIC become 0.025 μg mL^{-1} for both the bacteria.

Also in the LV case, the insertion of CNTs carries out to an enhancement of the FQs efficiency, with a reduction of the MIC from 0.10 (LV) and 0.40 (Gel_LV) to 0.025 μg mL^{-1} for *E. Coli*, and from 0.60 (LV) and 1.60 (Gel_LV) to 0.10 μg mL^{-1} for *K. Pneumoniae*.

Finally, when considering the hybrid materials containing LM as FQs, it should be highlighted that the presence of CNTs was able to enhance the performance of the Gel_LM conjugate against *E. coli* to a value similar to the free FQs, while its activity on *K. Pneumoniae* was not significantly modified.

Moreover, the activity of all the final hybrid composites makes possible a classification of both the Gram-bacteria as susceptible, with the sequence of antimicrobial activity was as follows: CNTs_Gel_LM < CNTs_Gel_LV = CNTs_Gel_CP for *E. Coli*, and CNTs_Gel_LM < CNTs_Gel_LV < CNTs_Gel_CP for *K. Pneumoniae*.

A further consideration could be done when considering the CNTs equivalent concentrations corresponding to the recorded MIC. By analyzing the data, it is clear that the combination between CNTs and LV is the most effective, since a very low amount of CNTs (0.17 and 0.69 μg mL^{-1}) was found able to increase the activity of free LV by four and six times against *E. Coli* and *K. Pneumoniae*, respectively. A higher amount of CNTs was required to enhance the free CP efficiency (1.56 μg mL^{-1} for both the bacteria). Finally, in the LM case, a comparable CNTs amount was enough to reach efficiency similar to the free FQs, while an even higher amount (5.56 μg mL^{-1}) did not affect the Gel_LM activity. Furthermore, for both the bacteria, the CNTs amounts corresponding to the recorded MIC values, were lower to those reported in literature for pristine CNTs [42], thus the enhanced antibacterial activity was related to the increased cell interaction and further internalization operated by CNTs.

The enhancement of the antibacterial activity was also evident when considering the MBC values, determined by plating 0.1 ml from the tubes showing no visible growth onto agar plates and incubating for 24h (Figure 6, Table 2).
The MBC values for the nanocomposites were found to be lower than that recorded for the free FQs and the Gel_FQs conjugates, except for CNTs_Gel_LM.

As explained for MIC, also in the MBC it was observed that a low amount of CNTs was required for the increase in the activity, confirming the suitability of the proposed approach for the preparation of highly effective antibacterial materials.

CONCLUSION

Hybrid materials with superior biocompatibility and enhanced antibacterial property were prepared by non-covalent functionalization of CNTs outer surface with Gel_FQs bioconjugates. Gel_FQs were obtained by free radical grafting of FQs on Gel, and CNTs subsequently introduced by sonication. The morphology of the samples showed the homogeneous dispersion of CNTs materials inside the protein conjugates and the whole system was found to be highly biocompatible, since no toxic affect were recorded on the in vitro model composed of hMSCs cells. The results of the antibacterial tests showed the suitability of the proposed strategy to match the key findings of all the counterparts: the high biocompatibility of Gel was, indeed, associated to the FQs antibacterial activity which was greatly enhanced by the presence of CNTs. These nanostructures carried out to more performing bacterial interaction and internalization of the FQs. The possibility to prepare highly performing antibacterial materials by using a very low amount of FQs and CNTs with no toxicity concerns is the key result of the proposed strategy, which could be further explored for practical applicability in biomedical field.

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Table 1. MIC values for tested compounds expressed as FQs and CNTs equivalent concentrations.

Code	FQs (μg mL^{-1})		CNT (μg mL^{-1})	
	E.Coli	*Klebsiella*	*E. Coli*	*Klebsiella*
CP	0.05	0.10		
Gel_CP	0.05	0.10		
CNTs_Gel_CP	0.025	0.025	1.56	1.56
LV	0.10	0.60		
Gel_LV	0.40	1.60		
CNTs_Gel_LV	0.025	0.10	0.17	0.69
LM	0.05	0.05		
Gel_LM	0.20	0.20		
CNTs_Gel_LM	0.05	0.20	1.39	5.56

Table 2. MBC values for tested compounds expressed as FQs and CNTs equivalent concentrations.

Code	FQs (μg mL^{-1})		CNT (μg mL^{-1})	
	E. Coli	*Klebsiella*	*E. Coli*	*Klebsiella*
CP	0.05	0.10		
Gel_CP	0.05	0.10		
CNTs_Gel_CP	0.025	0.05	1.56	3.12
LV	0.10	0.60		
Gel_LV	0.40	1.60		
CNTs_Gel_LV	0.05	0.20	0.34	1.38
LM	0.05	0.05		
Gel_LM	0.20	0.20		
CNTs_Gel_LM	0.10	0.20	2.78	5.56

Figure 1. Representative SEM (A) and TEM (B) images of pristine CNT used to assess the CNTs size properties; representative SEM images of CNTs_Gel_FQs (C,D), showing the presence of the protein network around the CNTs.

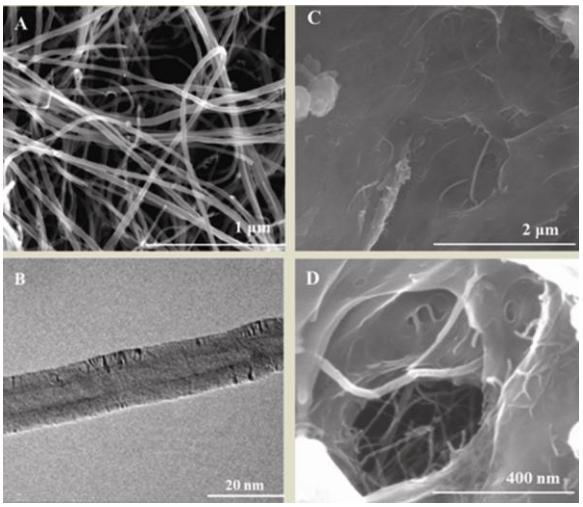
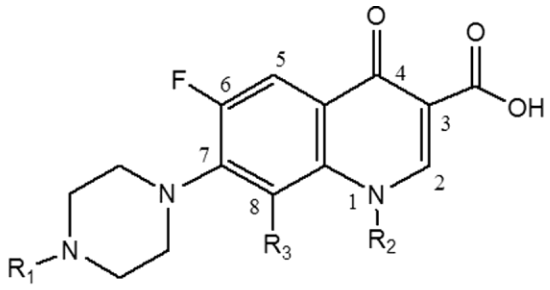


Figure 2. Chemical structures of employed FQs, highlighting the main position involved in the grafting reaction



FQs

CP: R_1= H; R_2= cyclopropil; R_3= H

LM: R_1= H; R_2= ethyl; R_3= F

LV: R_1= methyl; R_2 ⌣ R_3= $CH(CH_3)CH_2O$

Figure 3. FT-IR (A) and UV-Vis (B) absorption spectra for free and Gel-conjugated FQs.

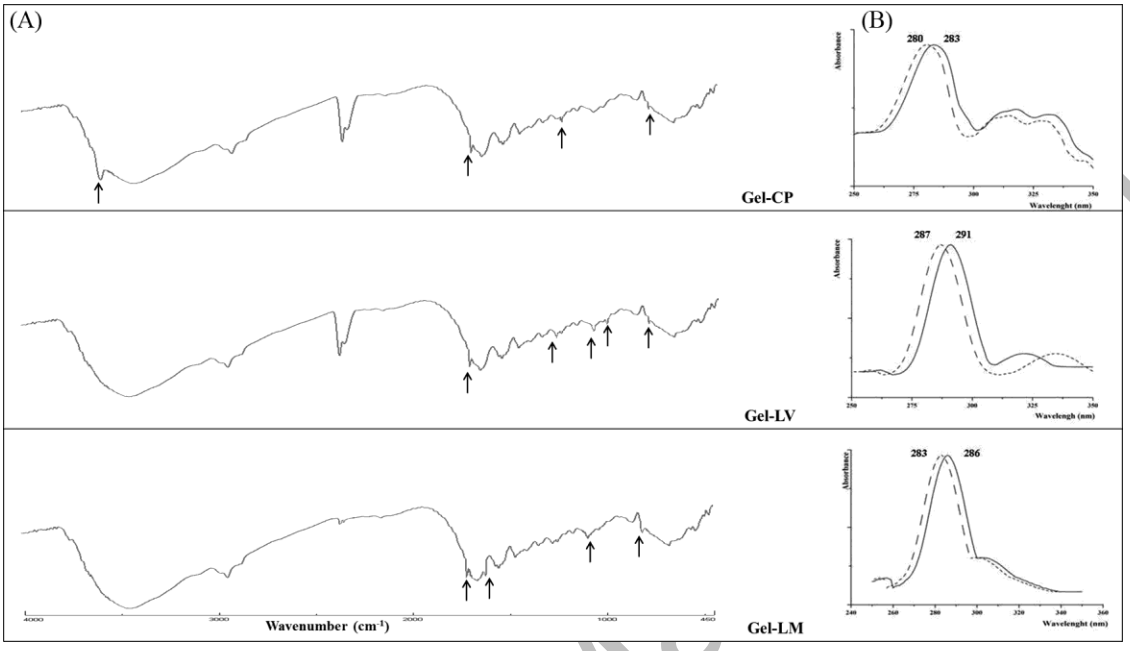


Figure 4. Schematic representation of the preparation of hybrid materials, highlighting the water dispersivity properties.

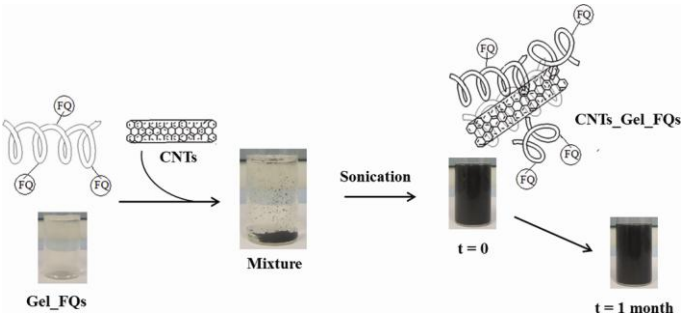


Figure 5. Representative image for MIC evaluation. CNTs_Gel_LV vs *Klebsiella Pneumoniae*. The FQs equivalent concentration are reported as μg mL^{-1}.

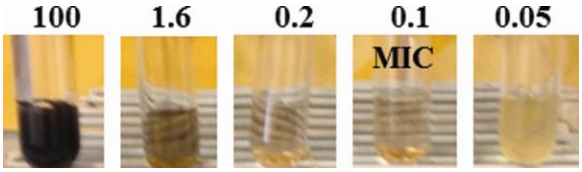


Figure 6. Representative image for MBC evaluation.