Antimicrobial and Antioxidant Supramolecular Ionic Liquid Gels from Biopolymer Mixtures

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Abstract

In this work, we describe the preparation and characterization of supramolecular ionic liquid gels based on binary mixtures of biopolymers, comprising chitosan, chitin, cellulose and lignin. The gels were obtained in ionic liquids differing for the cation or the anion, with no need for a cross-linking agent or acid treatment. The materials obtained were characterized for the minimum gelation concentration, porosity, swelling and rheological properties, finding a prominent influence of the nature of the ionic liquid anion. Then, we investigated the ability of the gels to scavenge free radicals,

finding that the gels exhibit a higher antioxidant ability than their separate components. Moreover, our gels showed a bactericidal activity toward Gram-positive and Gram-negative bacterial strains like *Streptomyces coelicolor* and *Escherichia coli*, respectively. Kinetics of bacterial cell viability showed that the gel based on a chitosan:chitin mixture in the IL [bmim][Cl] was the fastest acting against *E. coli*, with a practically total killing of bacterial cells within 30 min. Once again, a major influence of the ionic liquid anion was detected. Finally, we found an inverse relation between the efficacy of our gels to act as antioxidant or antimicrobial agents.

Introduction

Oxidation and microbial contamination, are phenomena of considerable concerns due to their negative impacts on human life. In particular, oxidation events are the main cause of food spoilage, and microbial contamination poses serious harm on human health when involving food or medical equipment. Therefore, it is highly desirable to devise and use materials endowed with inherent antioxidant and antibacterial ability. In this context, materials based on biopolymers, mostly those deriving from lignocellulosic biomass or marine organisms, have attracted significant interest in the last years.[1]

The advantage of biopolymers over synthetic antioxidant or antimicrobial agents lie in their biocompatibility, biodegradability and low toxicity as well as in being obtained from cheap, renewable sources. For this reasons, biopolymer-based materials have been employed in food packaging with antimicrobial or antioxidant activity.[2-5]

In addition, biopolymers in many instances impart to the ensuing materials a notable mechanical strength and resistance, allowing their use in tissue engineering like, for example bone substitutes.[6, 7] In this context, prominent examples of biopolymers used for these formulations include polysaccharides like chitin, chitosan and cellulose or a highly aromatic polymer like lignin. In particular, chitin is a main constituent of exoskeleton of crustaceans, and chitosan is a deacetylated

derivative of chitin. On the other hand, cellulose and lignin are by far the most abundant constituent fraction of lignocelllulosic biomass.

Among the various classes of antioxidant and antimicrobial materials in which biopolymer can be embedded, supramolecular gels[8-10] have recently gained considerable interest. Supramolecular gels are materials originating from the self-assembly of molecules called gelators which, in dilute solutions and under suitable conditions, aggregate spontaneously forming a sample-spanning fibrous network, able to entrap significant amounts of solvent by capillary forces. As a result, although the solvent constitutes the overwhelming majority in terms of mass, the resulting material has properties more similar to the ones of a solid. Supramolecular gels are underpinned solely by non-covalent interactions, and so their formation is usually reversible, and, in principle, they can respond reversibly to external stimuli. It is worth noting that, supramolecular gels are generally formed by small molecules, but they can also be based on polymers, provided that no covalent cross-linking among the chains occurs.[11]

Gels are usually classified based on the solvent in which they are prepared, and alongside the classic gels in water or organic solvents, known as hydro- and organogels, in the last years gels in non-conventional solvents have attracted increasing interest,[12] like the ones in deep eutectic solvents and ionic liquids, known as eutectogels[13] and ionic liquid gels,[14] respectively. In particular, ILs are especially attractive for the obtainment of biopolymer-based materials, given the high solubilizing ability of some of them towards polysaccharides[15-17] and lignin.[18] This is especially true for ILs bearing strong hydrogen bonding acceptor anions, which partially disrupt the network of intra- and intermolecular hydrogen bonds among polysaccharide chains, enhancing their solubilization.[19] Accordingly, ionic liquid gels of polysaccharides have been described, obtained from chitin,[20] cellulose[21] and chitosan.[22] Thanks to the properties of both polymers and ILs, such materials are easy to prepare and do not require the use of cross-linking agents or treatment with acids, as usually happens for biopolymer gels in conventional solvents. Recently, the possibility to obtain gels from

mixtures of biopolymers has revealed as a facile strategy to enhance or tune the mechanical properties of the ensuing gels.[23-25]

In the framework of our interest in studying the properties and applications of supramolecular gels in non-conventional solvents,[26, 27] we have recently demonstrated that such materials can present antimicrobial[28] and antioxidant properties.[29, 30]

Moreover, we also showed that polysaccharide-based ionic liquids gel can be successfully employed as sustainable materials for the desufurization of fuels.[25]

Based on all these considerations, we prepared and characterized ionic liquid gels obtained from binary mixtures of biopolymers like cellulose (CE), chitosan (CS), chitin (CT) and lignin (LG), as reported in Figure 1.

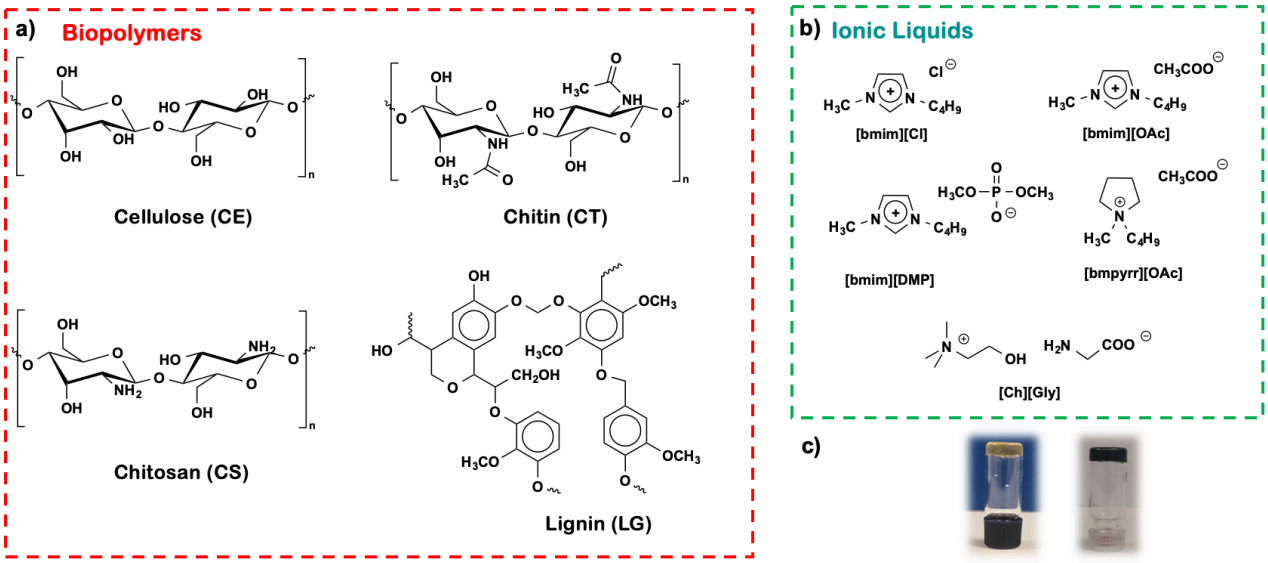

Figure 1. a) Biopolymers used, b) ionic liquids and c) representative pictures of ionic liquid gels obtained.

Firstly, we conducted preliminary gelation tests, obtaining gels in ILs differing for both cation and anion. More specifically, we used ILs bearing both aliphatic and aromatic cations, as well as anion with different coordination ability. When a gel was obtained, we also determined the minimum gelation concentration, *i.e.* the minimum amount of polymer mixtures required for gelation to occur, to evaluate and compare the gelation propensity of our mixtures. The mechanical properties of the

gels were investigated by oscillatory rheology, and characterization also involved the estimation of gel porosity and swelling upon contact with hexane. Subsequently, we evaluated the antioxidant ability of the gels and of their components taken separately, by the DPPH assay. Moreover, we investigated the antimicrobial activity of our gel towards *S. coelicolor* and *E. coli* as Gram-positive and Gram-negative tester strains, evaluating the possible bactericidal activity of our gels by kinetic measurements of bacterial cell viability.

All the gels showed high antioxidant and antibacterial activity, showing significant difference in the rate of action, with a prominent influence of the IL anion.

Experimental section

Materials

Cellulose, chitosan (low molecular weight), lignin (dealkaline) and chitin were obtained from commercial sources and used without further purification. Commercially available acetic acid, sodium hydroxide, methanol, hexane, diethyl ether, 1-butyl-1-methylpyrrolidinium chloride and Amberlyst IRA-400 (Cl^- form), were used as received. Cholinium chloride, 1-butyl-3-methyl-imidazolium acetate, [bmim][OAc]), 1-butyl-3-methyl-imidazolium dimethylphosphate, [bmim][DMP], and 1-butyl-3-methyl-imidazolium chloride, [bmim][Cl] were obtained from commercial sources and kept in a desiccator under argon and reduced pressure prior to use.

Cholinium glycinate[31] and 1-butyl-1-methylpyrrolidinium acetate[32] were prepared according to reported procedures.

Gelation tests

Solid mixtures of polymers were prepared by grinding in a mortar the suitable amounts of polymers, in the weight ratio 1:1, until obtaining a fine, uniform powder. Ionic liquid gels were prepared by weighing into a screw-capped vial (diameter = 1 cm) the suitable amounts of polymer mixture and IL. Depending on the nature of gel components, two different procedures were used. In particular, in

the first one, the mixture was sonicated for 5 min and then heated in an oil bath at 100 °C, until a clear solution was obtained. In the second one, the mixture was heated in the same way, without prior sonication. The procedure followed for each gel is reported in Table S1. The vial was then cooled and stored overnight at room temperature. Gel formation was then examined by the tube inversion test.[33]

Rheology measurements

Rheological measurements were carried out on a strain-controlled rheometer equipped with a Peltier temperature controller and a plate-plate tool. In a typical measurement, the gel was formed in a plastic blister pack, then transferred between the shearing plates of the rheometer. Strain and frequency sweep measurements were carried out at 25 °C, on three different aliquots of gels, within the linear viscoelastic region. In particular, strain sweeps were performed at a frequency of 1Hz, while frequency sweeps at a fixed oscillation strain of 1%.

Porosity and swelling

Porosity[34] and swelling[35] of all ionic liquid gels were determined following reported procedures, using HPLC-grade hexane as the solvent. Hexane was cast on gels for 24 h.

Knowing the initial weight of the empty vial (W_1), the weight of the vial and the gel before (W_{dry}) and after (W_2) adding hexane, together with the final weight of the vial and gel after removing hexane ($W_3 = W_{wet}$), it was possible to determine the porosity (P) and swelling (Q) using the following equations*:*

$$V_g = 4 - \left(\frac{W_2 - W_1 - W_{dry}}{\rho_h} \right) \quad \textbf{(1)}$$

$$V_p = \frac{W_2 - W_3 - W_{dry}}{\rho_h} \quad \textbf{(2)}$$

$$P = \frac{V_P}{V_p + V_g} \cdot 100 \quad \textbf{(3)}$$

$$Q = \frac{W_{wet} - W_{dry}}{W_{dry}} \cdot 100 \quad \textbf{(4)}$$

where ρ_h is the density of hexane, V_g and V_p (mL) represent the volume of gel and hexane in the pores, respectively. Finally, 4 mL is the total volume occupied by the gel and hexane.

DPPH assay

The DPPH free radical scavenging assay on gels was carried out following a reported procedure.[29, 30] Samples for a typical measurement were prepared by placing, in a screw-capped vial, 250 μL of a 10^{-3} M solution of DPPH in diethyl ether, on top of 250 mg of preformed gel. Contact between gel and solution was maintained for a suitable time, at 25 °C. All the gels were stable during this phase. Subsequently, the solvent was removed in vacuum and the residue was dissolved in 5 mL of methanol. Then the UV-vis of the solution was recorded. Another aliquot of DPPH solution, used as reference, was treated in the same way except for being in contact with the gel. The radical scavenging efficiency was determined by Equation (5)

$$SE\ (\%) = \frac{A_t - A_b}{A_b} \cdot 100 \quad \textbf{(5)}$$

where A_t is the absorbance of the sample solution and A_b is the absorbance of the reference solution, at the same time.

The DPPH assay on the single gel components was performed as described above, using the quantities of polymer or ILs present in 250 mg of gel.

Antimicrobial activity

Streptomyces coelicolor M145 and *Escherichia coli* DH10B were used as tester strains to assess antimicrobial activity. Specifically, an amount of 250 mg of preformed gel were transferred in the wells of a 24-multiwell plate and sterilized by irradiating with UV light for 15 min under a laminar flow microbiological hood. Subsequently, the wells containing the gel and 1 mL of R5A or LB growth medium were inoculated with *S. coelicolor* spore or *E. coli* cell suspensions at the final concentration of 10^7-10^8 CFU/ mL^{-1} . For both test strains, parallel bacterial cultures performed as described above except for the addition of the preformed gels or bacterial inoculates were used as positive and negative bacterial growth controls, respectively. The bacteria cultivations were incubated using an orbital shaking (170 rpm) at 30 °C for 5 days and for 24 h at 37 °C in the case of *S. coelicolor* and *E. coli*, respectively. Each cultivation was performed in triplicate.

Bacterial cell killing kinetics

The bactericidal activity of the gels was evaluated by performing cultures of *E. coli* cells exposed to gels as above described and sampling culture aliquots after 15, 30, 60 and 120 min of gel exposure. The different aliquots were then used to perform the counting of viable cells by serial dilution method using LB agar plates incubated overnight at 37 °C. The viable cells, revealed as CFU on LB-agar plates, were reported as percentage values in the respect of CFU from unexposed cultivations. Each cultivation was performed in triplicate.

IL release experiments

To 500 mg of preformed ionic liquid gel in a vial, 500 μg of D_2O were added, maintaining contact for a suitable time, at 25 °C. Subsequently, the supernatant was removed and added with 300 μL of a solution of maleic acid in D_2O (1.15 M). The ^1H NMR spectrum of the solution obtained was recorded, and the amount of IL released was determined based on integration of IL signals and the one of maleic acid as internal standard.

Results and discussion

Gelation tests

The polymer mixtures used as gelators were prepared by grinding in a mortar weighed amounts of two polymers until obtaining a uniform powder. Then, the suitable amounts of mixture were heated in the presence of the ILs. The mixture used were: CS:CE, CS:CT, CS:LG, CT:CE and CT:LG. In some cases, prior sonication was necessary to obtain a gel. The detailed information on the preparation protocols is reported in Table S1 while the results of the gelation tests are reported in Table 1.

Table 1. Outcome of gelation tests for biopolymer mixtures in ILs.

	CS:CE		**CS:CT**		**CS:LG**		**CT:CE**		**CT:LG**	
IL	**Range[a] (wt%)**	**App.[b]**	**Range[a] (wt%)**	**App.[b]**	**Range[a] (wt%)**	**App.[b]**	**Range[a] (wt%)**	**App.[b]**	**Range[a] (wt%)**	**App.[b]**
[bmim][Cl]			2.6:2.6	G	2.7:2.7-5.0:5.0	G	2.6:2.6-5.0:5.0	G	5.9:5.9	S
[bmpyrr][OAc]	5.0:5.0[c]	G[d]	5.0:5.0-9.7:9.7	G	4.7:4.7-9.6:9.6	G	9.9:9.9	S	6.0:6.0	S
[bmim][OAc]	5.0:5.0[c]	G[d]	5.0:5.0-9.8:9.8	G	5.0:5.0-9.9:9.9	G	9.9:9.9	PG		
[bmim][DMP]	2.7:2.7-4.9:4.9	G					9.8:9.8	PG		
[Ch][Gly]	5.2:5.2-9.3:9.3	S	5.0:5.0-9.4:9.4	PG	9.7:9.7	S	4.6:4.6	S		

[a] Mixture concentration range (wt%). [b] Appearance: S: soluble; G: gel; PG: gel-like precipitate. [c] MGC, Ref. 25. [d] Ref. 25.

Examination of result reported in Table 1 shows that most of the gels, originated by chitosan-containing mixtures. The only gel obtained from a mixture without chitosan is the one of CT:CE in [bmim][Cl]. No gels were obtained from the CT:LG mixtures, in the ILs considered or in solution of [Ch][Gly] for all the mixtures used due to the higher solubility of polymers in this IL.

To better estimate the propensity of each polymer mixture to form ionic liquid gels, we determined, for all the gels obtained, the Minimum Gelation Concentration (MGC), *i.e.* the minimum amount of gelator, polymer mixture in this case, required to obtain a gel. The results obtained are reported in Figure 2.

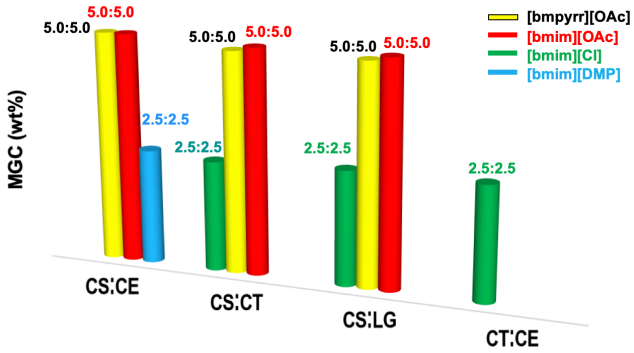

Figure 2. MGCs for biopolymer-mixtures gels in ILs. Values for CS:CE/[bmim][OAc] and CS:CE/[bmpyrr][OAc] are taken from Ref. 25.

A first look at the results reported in Figure 2, clearly shows that the MGC values are mainly influenced by the IL, and the effect of changing mixtures is rather marginal. Indeed, IL being the same, no variations in MGC are found by changing polymer mixture. A more detailed picture on the influence exerted by the IL nature on MGC, emerges by considering the influence of IL cation and IL anion, separately. In particular, considering ILs differing for the cation such as [bmpyrr][OAc] and [bmim][OAc], MGC has practically the same value, irrespective of the mixture considered. Conversely, the nature of the IL anion has much more significant influence. Considering [bmim$^+$]-based ILs, MGC increases on going from [bmim][Cl] to [bmim][OAc] in the presence of CS:CT and CS:LG, and from [bmim][DMP] to [bmim][OAc][25] in the presence of CS:CE. In all cases the trend of MGC parallels the one of the hydrogen bonding accepting ability of the anion, as estimated by the

Kamlet-Taft paramter β, which varies along the sequence $[Cl^-] < [DMP^-] \approx [OAc]$ (β = 0.87, 0.96 and 1.05 for [bmim][Cl],[36] [bmim][DMP][37] and [bmim][OAc],[38] respectively).

This suggests that gelation of our mixtures is favored in ILs with lower hydrogen bonding accepting ability. We propose that stronger hydrogen bond acceptor anions interfere with the establishment of hydrogen bonding network between polymer chains. In other words, a strongly hydrogen bonding donating polymer, will be more solvated in the presence of a strongly coordinating anions, and therefore less available to interact with other polymer chains.

Then, we determined the porosity (P) and swelling (Q) of our gels by contacting them, for 24 h, with hexane (see experimental section for details). From now on, the characterization discussed refers to gels at the common concentration of 5:5 wt% Results obtained using reported procedures,[35, 39] are summarized in Table 2.

Table 2. Swelling and porosity determined upon contact of ionic liquid gels with hexane.

Entry	Gel	Q (%)	P (%)
1	CS:CT/[bmim][Cl]	13	33
2	CS:CT/[bmim][OAc]	-	56
3	CS:CT/[bmpyrr][OAc]	-	40
4	CS:CE/[bmim][DMP]	21	30
5	CT:CE/[bmim][Cl]	-	13
6	CS:LG/[bmim][OAc]	-	44
7	CS:LG/[bmim][Cl]	12	46
8	CS:LG/[bmpyrr][OAc]	10	7

In general, the swelling of the gels in contact with hexane was rather low or negligible, with the only exception of the CS:CE/[bmim][DMP] gel for which a swelling of 21% was detected. Moving to porosity, in general all the biopolymer-based ionic liquid gels, exhibited low to average porosity.

Also in this case, we examine the effects of IL ions on this parameter. In particular, switching from the aliphatic cation of [bmpyrr][OAc] to the aromatic one in [bmim][OAc], induces a significant increase in porosity in the case of CS:CT (entries 2 and 3) and CS:LG (entries 6 and 8). Interestingly, polymeric mixtures featured by a weaker hydrogen bond, such as CS:LG, feels more the effect of the IL cation structure. In particular, the results obtained can indicate that cation-π interactions occurring between the aromatic polymer, LG, and the [bmpyrr$^+$] cation give rise to the formation of a denser network compared with the one occurring for LG in [bmim$^+$]-based ILs, in which π-π interactions should mainly operate.

On the other hand, the effect of the anion is more articulate. In particular, in the case of CS:CT-based gels, porosity increases on going from [bmim][Cl] to [bmim][OAc], in parallel with the increase in the anion coordination ability. Conversely, comparable values are obtained in the case of CS:LG-based gels in the same ionic liquids. Once again, the trend obtained evidences that the solvent effect is, in turn, dependent on the nature of the polymeric mixture, with the one formed by stronger hydrogen bond donors feeling more the effect of the IL anion.

To investigate the mechanical properties of the gels, we carried out rheological measurements such as strain- and frequency sweeps, at 25 °C. We chose some representative gels, to evaluate the IL anion and cation effect with a common polymer mixture, as well as the effect of changing polymers, for gels sharing the same IL. In these measurements, the evolution of two parameters, such as G′, the storage modulus, relating to solid-like rheological response, and G″, loss modulus, relating to liquid-like rheological response, is followed as a function of the applied strain and frequency. Another important rheological parameter that can be obtained from such measurements is the strain at crossover point (γ_c) which represents the maximum strain tolerated by the gel before breaking. The results obtained are reported in Figure 3 and Figure S1, while the rheological parameters are summarized in Table 3.

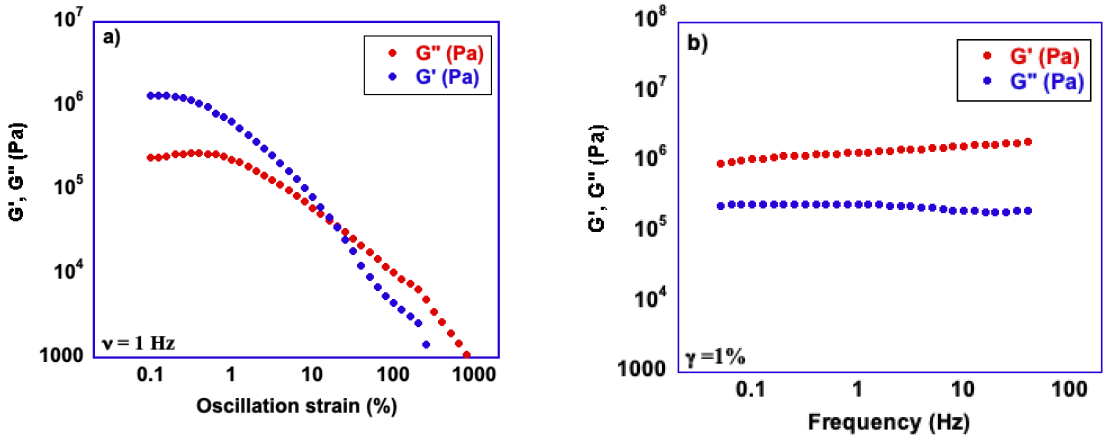

Figure 3. a) Strain- and b) frequency sweeps for the CS:LG/[bmim][Cl] 5:5wt% at 25 °C.

Table 3. Rheological parameters for biopolymer-based ionic liquid gels.

Gel	G′ (Pa)	G″ (Pa)	tan δ	γ_c (%)
CS:CT/[bmim][Cl]	$(6.7 \pm 0.4) \cdot 10^3$	$(2.8 \pm 0.3) \cdot 10^3$	0.41 ± 0.02	12.46 ± 0.09
CT:CE/[bmim][Cl]	$(4 \pm 1) \cdot 10^3$	$(2.0 \pm 0.2) \cdot 10^3$	0.6 ± 0.3	8 ± 2
CS:LG/[bmim][Cl]	$(1.0 \pm 0.3) \cdot 10^6$	$(2.4 \pm 0.1) \cdot 10^5$	0.28 ± 0.09	20 ± 3
CS:LG/[bmim][OAc]	$(1.4 \pm 0.1) \cdot 10^3$	480 ± 50	0.35 ± 0.06	700 ± 100
CS:CE/[bmim][OAc] [25]	$(3.3 \pm 0.5) \cdot 10^3$	$(1.2 \pm 0.3) \cdot 10^3$	0.40 ± 0.09	20 ± 6
CS:CE/[bmpyrr][OAc][25]	$(4.2 \pm 0.9) \cdot 10^3$	$(8.5 \pm 1.8) \cdot 10^2$	0.205 ± 0.004	49.9 ± 0.4

The results reported in Table 3 show that in all cases, tan δ, i.e. the ratio G″/ G′ is lower than 1, which reveals dominance of solid-like behavior and the occurrence of strong colloidal forces within the gel network. A closer look on the trend of G′, once again reveals a prominent influence of the IL anion. In particular, for the CS:LG-based gels, the storage modulus G′ is much higher for the one obtained in the IL [bmim][Cl], compared with the one obtained in [bmim][OAc]. In other words, the presence of the less coordinating chloride anion in the IL leads to the obtainment of the stiffest gels.

An opposite picture is found when we consider the variation of the strain at the crossover point, γ_c, as a function of the IL anion. In this case, the highest γ_c value is found for the gel formed in the [bmim][OAc^-]. Bearing in mind that γ_c expresses the maximum amount of strain that a gel can withstand without breaking, this suggests that gel formed in this ILs is the most flexible one.

Analyzing the effect of the polymeric mixture, the ionic liquid gels of formed in [bmim][Cl] and containing chitin, are sensibly weaker gels than the one formed by CS:LG, as evidenced by the higher tanδ values. More specifically, the gel obtained in the presence of CT:CE is the weakest one, with a more pronounced dependency of the modules on the frequency. In other words, the presence of chitin appears to exert a detrimental effect on the mechanical properties of the gels. This effect is likely due to the presence of acetylated groups in the backbone of chitin, that substantially lowers hydrogen bonding ability, with respect to chitosan and cellulose. Thus, substituting chitosan or cellulose chains with chitin ones, weakens the interchain hydrogen-bonding network which underpins the gels[24] and chitin is indeed known to form weak gels in imidazolium ILs.[40]

Antioxidant ability

The radical scavenging efficiency of our materials was determined by a widely used method, such as the DPPH assay.[41, 42] In this assay, the scavenging of the relatively stable 2,2-diphenyl-1-picrylhydrazyl radical, by and antioxidant species can be easily followed spectrophotometrically.

Initially, we set out to determine the antioxidant ability of the single gel components, *i.e.* polymer mixtures and ILs, taken separately. To have a meaningful comparison with the gels, in this assay we employed the same amounts of polymer and ILs present in the gels. In particular, contact between DPPH and gel components was maintained for 4h at 25 °C, obtaining the results reported in Figure 4. It is worth noting, that in the case of polymers we could not measure the scavenging efficiency of the lignin-containing mixtures, due to the partial dissolution of lignin, which covered the DPPH signal in the spectrum.

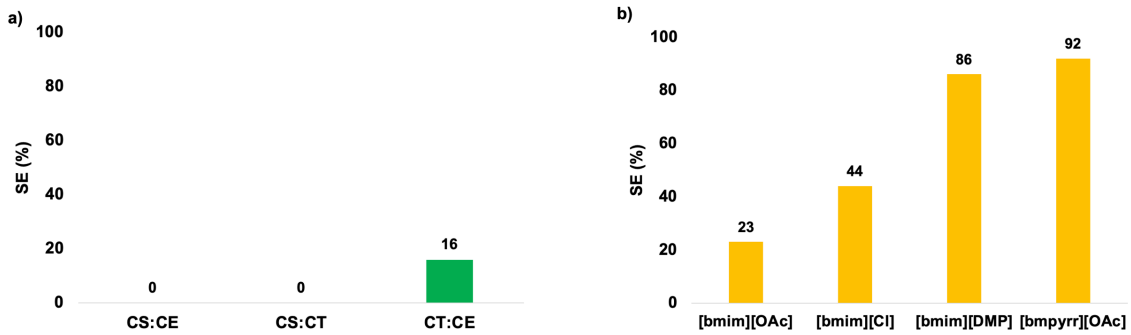

Figure 4. Antioxidant ability of a) polymer mixtures and b) ionic liquids, measured at 25 °C after 4h. SE% are reproducible within ± 4%.

The results reported in Figure 4a clearly show that the polymer mixtures used have negligible or only modest antioxidant ability. It is worth noting that this result does not derive from the insolubility of the polymer in the solvent used for the DPPH solution, since insoluble materials like carbon nanotubes, are fully capable of scavenging radicals, as reported in the literature.[29, 43]

A different picture emerges when we compare the results obtained with ILs, taken alone, reported in Figure 4b. In this case, ILs show significant antioxidant activity, and in two cases, namely [bmim][DMP] and [bmpyrr][OAc], they remove DPPH radicals almost completely. For this reason, we will not consider the antioxidant ability of gel obtained in these latter ILs, in the following discussion.

Subsequently, we determined the scavenging efficiency of our gels, in the same way described for their components. In this case, we carried out the assay at different times, comprised between 15 min and 4 h. The results obtained are reported in Figure 5.

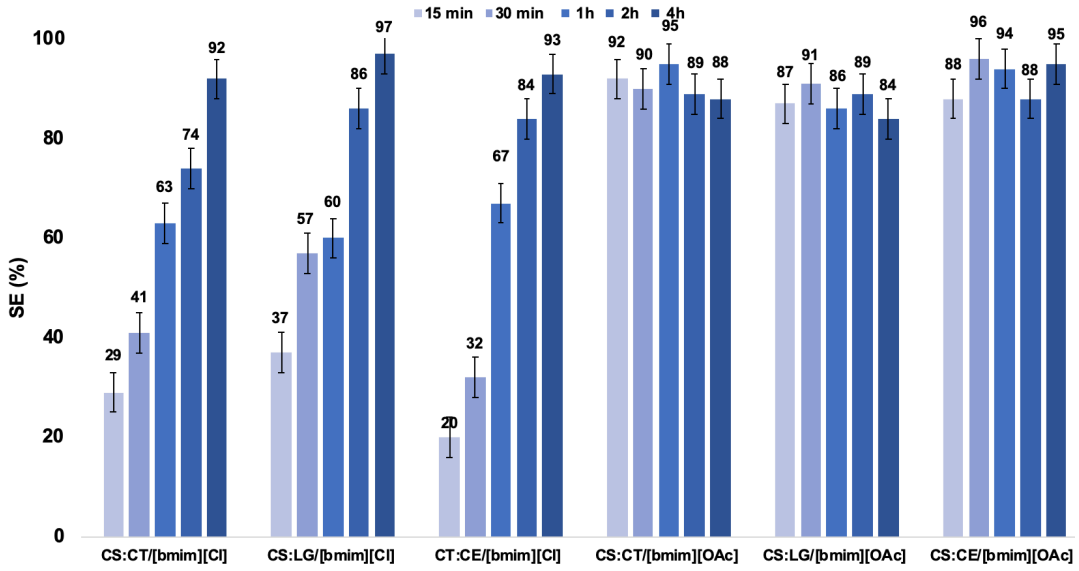

Figure 5. Scavenging efficiency of biopolymer-based ionic liquid gels at variable times.

Results reported in Figure 5 clearly show that the gels exhibit high scavenging efficiency, and are capable of removing the DPPH radical from the solution almost entirely. In all cases, scavenging efficiency of the gels is significantly higher than the ones of the single components, after the same time. A closer look shows a sharp difference, with some of the gels acting faster. In particular, a marked dependence on the nature of the IL anion clearly emerges, as the gels obtained in [bmim][OAc] scavenge the DPPH radical faster than those formed in [bmim][Cl], regardless of the polymer mixture used. It is worth noting that such result does not derive from the scavenging efficiency of [bmim][OAc] itself, because it was inferior to that of [bmim][Cl] considered alone, as reported in Figure 4b. This effect only manifests in gels, in which almost complete quenching of the DPPH radical is found after only 15 min. With the only exception of the CS:LG mixture, this result can be related to the porosity of the gels, since gels with higher porosity, such as CS:CT/[bmim][OAc], CS:LG/[bmim][OAc] and CS:CE/[bmim][OAc][25] exhibited also the highest scavenging efficiency as reported in Table 2. On the other hand, IL being the same, we observe no obvious influence of the nature of the polymer mixture used.

Notably, immobilizing the polymer mixture within the gel matrix, minimized leaching of the polymers. In particular, although the DPPH assay could not be carried out for the CS:LG mixture, due to the partial solubilization or suspension of lignin in ether, no leaching was detected for the CS:LG-based gel.

To find whether the radical scavenging efficiency of our gels comes only from the ILs or alternatively the biopolymers concur to the observed results, we also investigated the effect of polymer concentration by determining the time-dependent scavenging efficiency for CS:LG/[bmim][Cl] at 2.5:2.5 wt% instead of 5:5wt%. This gel was chosen because the MGC allowed it to be formed at a concentration significantly lower that 5:5 wt%. Moreover, since the variation of SE% as a function of time is slower than the [bmim][OAc]-based gels, any effect of concentration would be easier to follow. The results obtained are reported in Figure 6.

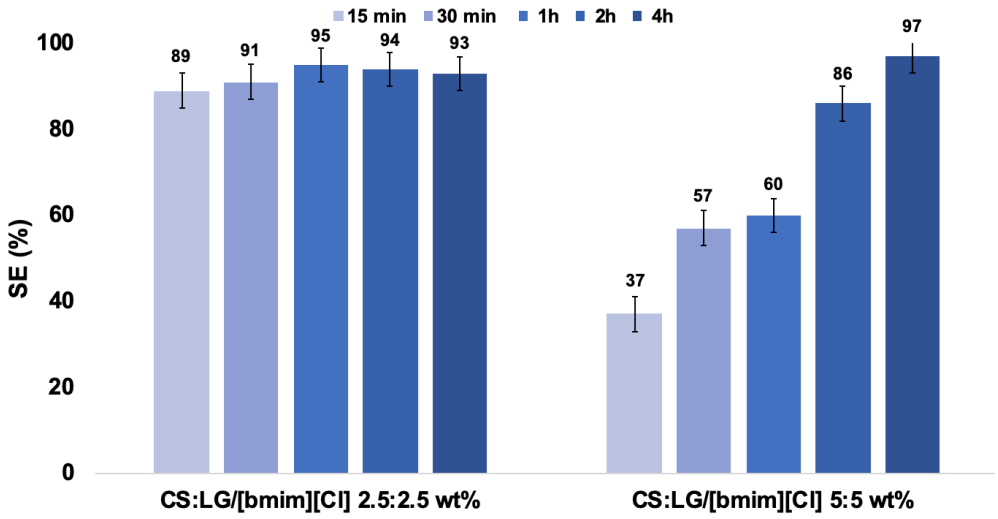

Figure 6. Scavenging efficiency of CS:LG/[bmim][Cl] at different concentrations.

The results reported in Figure 6 show that reducing the concentration of polymers, induces a sharp increase in the rate of scavenging. This result, that may appear surprising, is similar to other observations reported in the literature[44, 45] on the concentration-dependent behavior of different natural antioxidants, which, on increasing concentrations become less efficient in scavenging and in

some cases even become pro-oxidant.[46, 47] Despite being observed repeatedly, the precise reasons for such phenomenon are not yet fully understood. It is worth noting that in our case the gels maintain the ability to remove the DPPH radical almost entirely and the only difference is a kinetic one. This suggest that varying the amount of polymers on the gels can be a simple way to tune the rate of action, with an increase in concentration yielding materials better suited to a more prolonged antioxidant action. Based on these findings, we suggest that the antioxidant efficiency of the gels results from an interplay of polymers and IL alike.

Antimicrobial activity

The antimicrobial activity of our ionic liquid gels was initially investigated against two bacterial strains, the Gram-positive *Streptomyces coelicolor* and the Gram-negative *Escherichia coli*. Both strains are widely used as models for antimicrobial activity studies.[48-50] We chose to conduct these investigations on gels which resisted unaltered to contact with water for 24 h. In particular, these gels are: CS:CE/[bmim][DMP], CS:CT/[bmim][OAc], CS:CT/[bmim][Cl] and CT:CE/[bmim][Cl]. We also included the ionic liquid gels CS:CE/[bmim][OAc] and CS:CE/[bmpyrr][OAc], described in a previous work.[25]. In these experiments, 250 mg of preformed sterilized gel was placed in the wells of 24-multiwell plates containing 1 mL of R5A or LB in the case of *S. coelicolor* and *E. coli*, respectively. Then, the tester strains were inoculated at the final concentration of 10^7-10^8 CFU/ mL^{-1} spore o cell suspensions. The growth of the microbial cells was verified and compared using as positive and negative bacterial growth controls parallel bacterial cultures performed as described above except for the addition of the gels or of bacterial inoculates, respectively. Gels were then incubated for a period of 24 h and 5 days, at temperatures of 30 °C or 37 °C, depending on the tester strain considered (see experimental section for details). Pictures relevant to these experiments are reported in Figure 7.

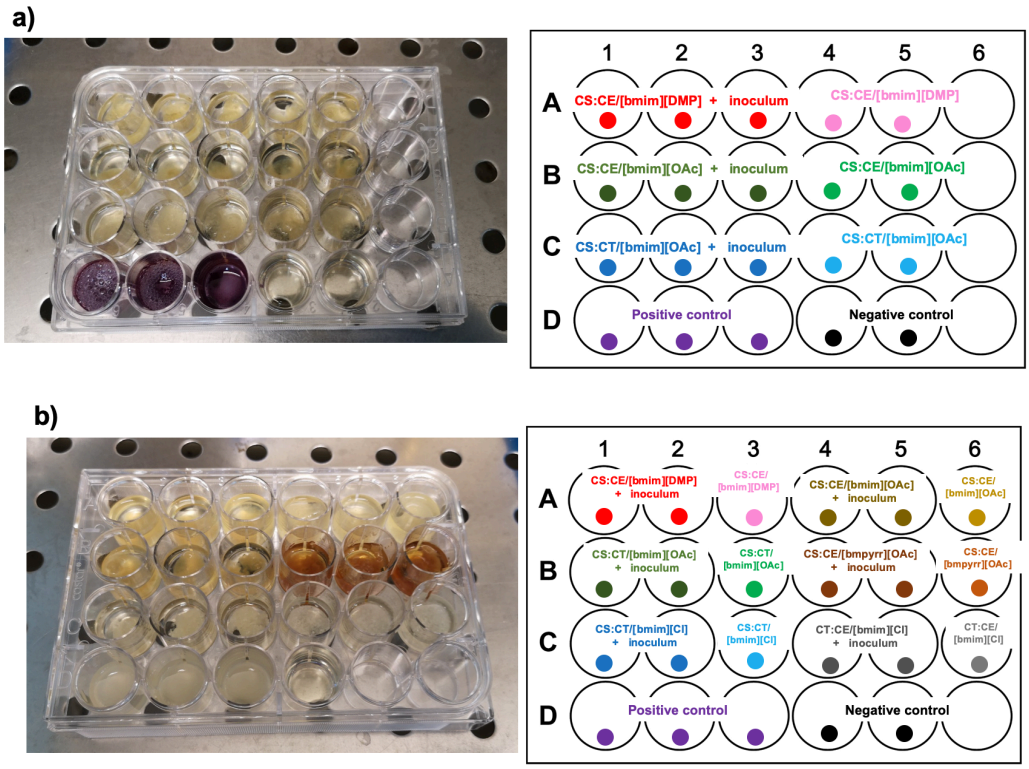

Figure 7. Pictures relevant to antimicrobial assays of gels against a) *S. coelicolor* and b) *E. coli*.

The results obtained in comparison with the control samples evidenced that in all cases the gels exert a significant antibacterial activity inhibiting bacterial growth. To further investigate the antimicrobial activity, *E. coli* cultivations exposed to gels were used in order to evaluate the bacterial cell viability by the method of serial dilution and CFU counting on LB-agar plates. The results revealed the absence of bacterial colonies from the gel-exposed samples, whereas bacterial colonies grew from the unexposed samples. Representative pictures are reported in Figure S2. These results reveal that the gels exert a bactericidal activity.

To further investigate the bactericidal action of our gels, we analyzed the bacterial viability kinetics by exposing to 250 mg gels *E. coli* cells (10^7-10^8 CFU/mL) incubated in 1 mL of LB culture medium placed in wells of 24-multiwell plates. Aliquots of the exposed cultivations were collected at different times. Then, the fraction of viable bacterial cells was determined in the respect of unexposed cultivations and plotted as a function of time, obtaining the results reported in Figure 8.

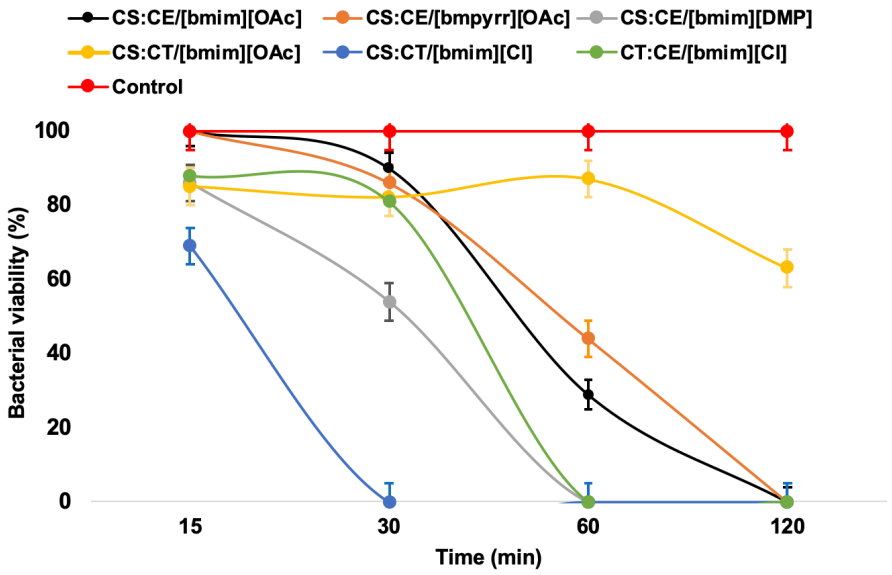

Figure 8. Bacterial viability kinetics (%) for biopolymer-based ionic liquid gels against *E.* Coli as tester strain. Lines are drawn as mere visual guides.

Looking at the results reported in Figure 8, brings out significant differences among gels in terms of the rate of bactericidal effect. In general, with the only exception of CS:CT/[bmim][OAc], all gels are capable of entirely killing all bacterial cell in at most 2 h. The fastest acting gel appears to be CS:CT/[bmim][Cl], in which case total elimination of bacterial viability is achieved in only 30 min. At the other end of the spectrum, the slowest bactericidal activity is observed for CS:CT/[bmim][OAc], in which case a reduction of only 37% in bacterial viability occurred after 2 h. Furthermore, the results obtained for the gels formed by CS:CE in [bmim][OAc] and [bmpyrr][OAc] show very similar kinetic profiles, suggesting negligible influence of the IL cation in the rate of bactericidal effect. Conversely, the IL anion exerts a much more significant influence, as can be seen by comparing gels sharing the same polymer mixture. In particular CS:CE/[bmim][DMP] acted consistently faster than CS:CE/[bmim][OAc]. The same holds true by comparing the killing kinetics of CS:CT/[bmim][Cl] and CS:CT/[bmim][OAc], indicating that antibacterial activity inversely parallel the gel porosity, which as reported in Table 2, amounts to 30%, 84%, 33% and 56% for

CS:CE/[bmim][DMP], CS:CE/[bmim][OAc], CS:CT/[bmim][Cl] and CS:CT/[bmim][OAc], respectively. Furthermore, this trend is opposite to the one found for antioxidant activity.

Finally, the nature of the polymer constituting the gels appear to play a role. More specifically, for [bmim][OAc]-based gels, CS:CE appears to induce a faster reduction of viability than CS:CT, and the same can be said comparing CS:CT and CT:CE for gels obtained in [bmim][Cl]. The whole of these findings suggests a concomitant role exerted by the nature of IL and polymers in eliciting the bactericidal effect. The relevance of these parameters is directly linked to the structural features that they impart to the gel phases. On the other hand, the increase in antimicrobial activity upon gelation has been reported for supramolecular gels formed by low molecular weight compounds, such as organic salts[28] and amphiphiles,[51] and has been linked to the supramolecular structure allowing the establishment of additional hydrogen bonding interactions with the bacterial cell wall.

To check for the possible release of gel components during contact with bacterial cells, we put in contact 500 mg of ionic liquid gels with 500 μL of D_2O as a model for the culture medium. After different times of contact, comprised between 15 and 60 min, we recorded the ^{1}H-NMR spectra of the supernatant aqueous phase, adding a known amount of maleic acid as internal reference.

This investigation was carried using as gels CS:CT/[bmim][Cl], CT:CE/[bmim][Cl] and CS:CE/[bmim][DMP], which were the fastest acting against *E. Coli* cells. Similarly, the time interval was the one in which these gels to entirely kill the bacterial cells. A representative spectrum is reported in Figure 9. The other spectra are reported in Figure S3, while the amounts of IL released are reported in Table 4.

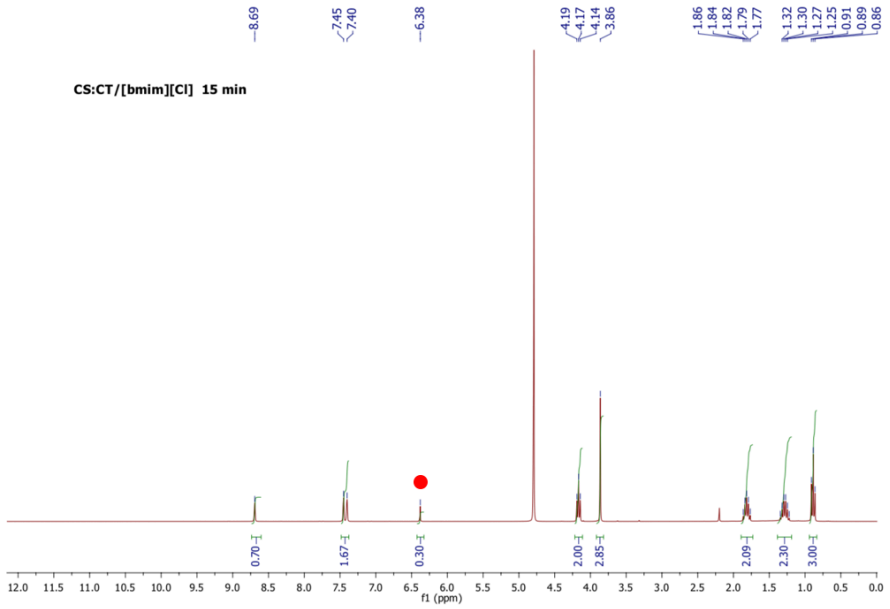

Figure 9. [1]H NMR spectrum obtained upon contact of CS:CT/[bmim][Cl] with D_2O after 15 min. The peak relevant to the reference compound, maleic acid, is highlighted.

Table 4. Mass of IL released as a function of time upon contact between ionic liquid gels and D_2O.

GEL	Time (min)	IL Mass released (mg)	% Mass released
CS:CT/[bmim][Cl]	15	23.7	5.2
	30	39.0	8.6
	60	45.1	10.0
CT:CE/[bmim][Cl]	15	22.8	5.1
	30	42.6	9.5
	60	44.0	9.8
CS:CE/[bmim][DMP]	15	44.3	9.7
	30	57.3	12.7
	60	76.1	16.9

Analysis of the NMR spectra obtained, reveals that in all cases only the IL is released form the gels, and no obvious evidence of signals relevant to the polymers was observed.

On the other hand, looking at the results reported in Table 4 shows that for all the gels considered, contact with D_2O induced the release of significant amounts of ILs. More specifically, the amount of ILs released depended mainly on the solvent used, as shown by the comparable results obtained for CS:CT/[bmim][Cl] and CT:CE/[bmim][Cl]. The magnitude of release of IL was higher for CS:CE/[bmim][DMP], which amounted to 17% in mass after 1h, while in the case of the [bmim][Cl]-based gels it amounted to 10%. The release of IL has been linked to the antimicrobial activity of different IL-based materials, such as supramolecular gels,[28] cryogels[52] and polymer blends.[53] Based on the results reported in Table 4, we cannot rule out a contribution of the IL released on the antimicrobial effect observed since bactericidal activity of imidazolium salts has been already described.[54, 55] However, the significantly different kinetic profiles shown by CS:CT/[bmim][Cl] and CT:CE/[bmim][Cl], despite the similar release values, suggest that the antimicrobial efficacy of our materials derives from a concomitant influence of both the IL and the polymers immobilized within the gel matrix.

Finally, a comparison of the results obtained for the gels in terms of antimicrobial and antioxidant activity, brings out a dichotomic behavior. In particular, gels that act faster as antibacterial agents tend to scavenge radicals in a relatively slower way and vice versa. This is exemplified by a gel like CT:CE/[bmim][Cl] which is the fastest acting against *E. coli* and lies on the slower end regarding the radical scavenging ability, as can be seen in Figure 5. Conversely, the slowest gel in terms of antibacterial activity, CS:CT/[bmim][OAc], is among the faster antioxidants among the gels considered. These findings can be explained considering that, as reported in the literature, oxidation stress events are involved in the processes leading to the disruption of viability of bacterial cells.[56, 57] Accordingly, gel that are faster and more effective in removing oxidant species, will exert this effect also towards the oxidation events involved in antibacterial activity, resulting in an overall slower antibacterial action.

Conclusions

We obtained supramolecular gels based on biopolymer mixtures, in ionic liquids. Characterization of the materials obtained in terms of Minimum Gelation Concentration, swelling and porosity as well as rheological properties showed a prominent influence of the ionic liquid anion on such parameters. In particular, gelation was favored in ILs bearing stronger hydrogen bond accepting anions. This property also led to the obtainment of more flexible gels as shown by the ones obtained in ILs bearing the acetate anion. On the other hand, gels obtained in the IL [bmim][Cl] emerged as the most rigid ones. All of our gels showed radical scavenging activity, significantly higher than the one their separate components. Also in this case, a prominent influence of the nature of the IL anion was detected, and in particular gels in [OAc^-]-based ILs acted faster than those obtained in [bmim][Cl]. Interestingly, the antioxidant activity of the gel phases could be related to their porosity, with more porous gels acting faster as antioxidants. We also investigated the antimicrobial activity of our gels, finding that they exert a bactericidal effect against *S. coelicolor* and *E. coli*, used as Gram-positive and Gram-negative tester strains. Kinetics of bacterial cell viability revealed that the gel CS:CT/[bmim][Cl] is the most effective, since it is able to kill all bacterial cells of *E. coli* in 30 minutes under the experimental condition used. Once again, biological activity can be ascribed to the nature of gel components which, impart the material properties like porosity which appear signifivantly linked to the rate of antibacterial activity. To obtain further information of the antibacterial activity of the gels, we estimated the release of IL in D_2O, as model for the aqueous culture medium, finding that significant amounts of IL are released in the aqueous phase. This evidence, together with the ones gathered by the killing kinetics experiments, suggested that the incorporation of the biopolymers within the gel matrix is essential to elicit the bactericidal activity and that a concomitant role of the IL can be hypothesized. Finally, an inverse relation between the rate of radical scavenging and antimicrobial activities of the gels was observed, explained by the possible involvement of oxidative stress events in the bactericidal action.

Conflict of interest: There are no conflicts to declare

Acknowledgements

We thank University of Palermo for funding (FFR 2021 D'Anna and FFR 2021 Marullo).

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