When the functionalisation comes in useful: ionic liquids with a "sweet" appended moiety demonstrate drastically reduced toxicological effects

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ABSTRACT. Growing applications of ionic liquids (ILs) in the industry have raised attention towards green credentials of synthesis, as well as their cyto- and ecotoxicities both for their use and accidental leakage into the environment.

With the above premises in mind, here we designed the ILs bearing aliphatic side chains or the anion incorporating gluconic acid (derived from food waste) moiety. An ionic liquid with imidazolium cation with an appended gluconic acid (bearing 5 hydroxyl groups) moiety was also synthesised for a useful comparison.

Different structural features were considered, like the nature and length of the alkyl chain and the nature of the anion. For comparison, two ILs with one hydroxy group and without any hydroxy-groups were prepared.

Apart from typical characterisation of ILs, such as differential scanning calorimetry (DSC), thermal gravimetric analysis (TGA), conductivity and viscosity measurements, ILs were also evaluated for their cyto- and ecotoxicities, performing tests on three cell cancer lines (HeLa, HTC-116 and MCF-7) and fish embryos (Zebrafish).

Data obtained shed light on the relationship working between physico-chemical properties and structural features of ILs. Interestingly, these ILs are able to inhibit cell growth only at very high concentrations ($IC_{50} \sim 10^{-3}$ M) and they aren't able to affect vitality of fish embryos, allowing to classify them as harmless solvents.

To our surprise, data collected shows that derivatisation with the "sweet" residue on the imidazolium cation completely removes well known toxicity effects of imidazolium ILs.

INTRODUCTION

Ionic liquids (ILs), organic salts with melting temperatures lower than 100 °C,[1] have been considered for a long time as an advantageous alternative to the use of conventional organic solvents. Their high thermal stability, low vapor pressure and flammability favoured their application as solvents,[2] catalysts,[3] and reagents.[4] As more applications of ILs appear, there is also a growing attention to their biodegradability and toxicity.[5-9]

Since a large number of ILs are water soluble, they can persist in the environment and interact with living organisms.[10-11] Furthermore, their degradation and persistence in the environment are heavily dependent both on the cation and anion structure.[10] As a consequence, over the past decade,

the emphasis on the design and synthesis of ILs focussing on the greener aspects of ILs is strong.[12-15]

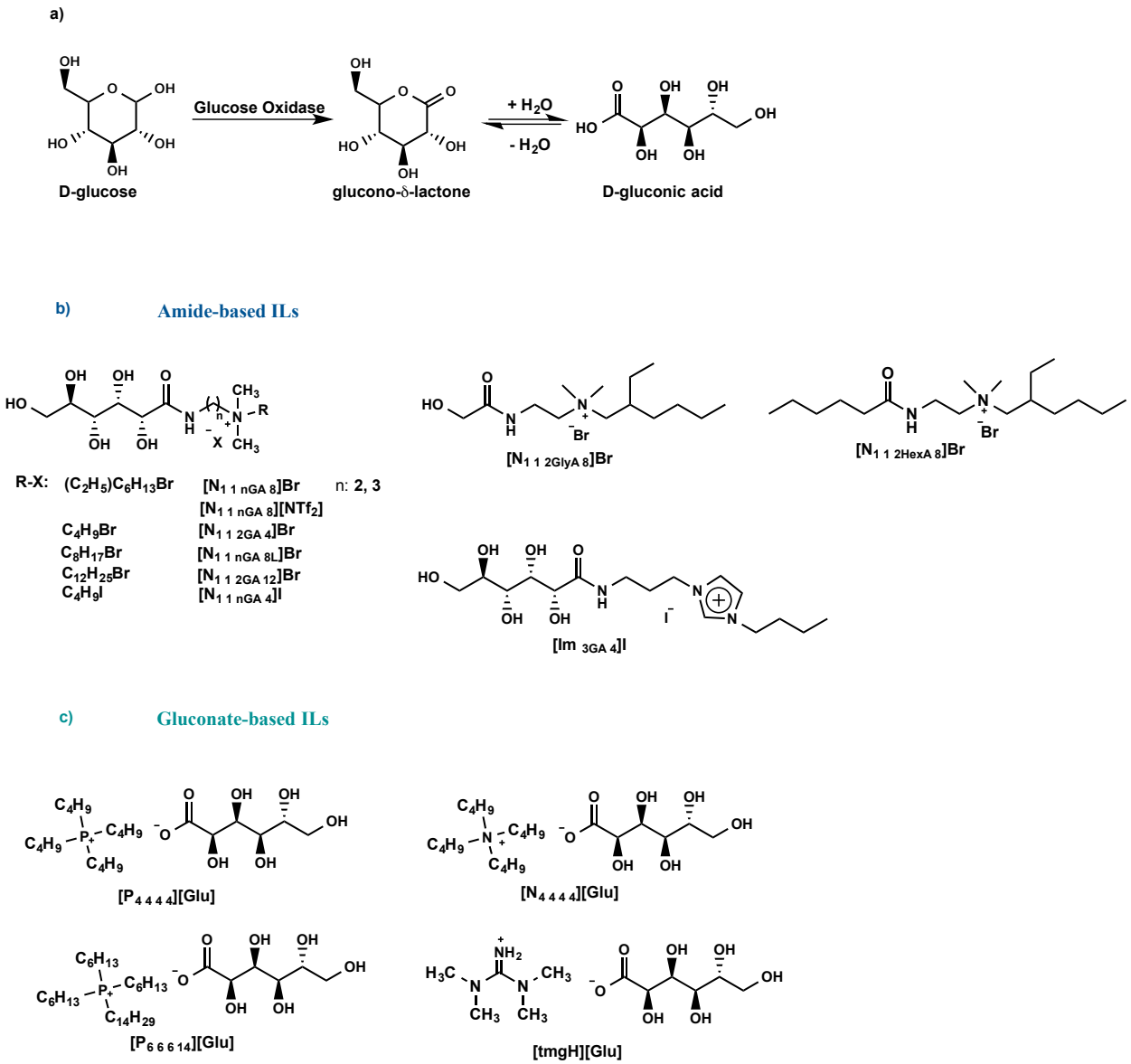
Among factors, the nature of the cation, length of the alkyl chain attached to it and the presence of functional groups on the chain play a significant role in determining the toxicity and biodegradability.[6,16]

In particular, biodegradation and toxicology investigations highlighted that chemical functionalities like hydroxyl, carboxyl and ester groups if inserted in the IL structure could make less harmful them towards the ecosystem.[17,18]

Aliphatic cations are usually more biodegradable and less toxic than the corresponding aromatic ones[19] and, among the former, phosphonium ILs may show toxicity similar to the imidazolium ILs.[20]

Imidazolium ILs are probably the most widely used from an industrial point of view. To name a few, BASIL™ and CELLIONIC™ processes, as well as their utilisation in lithium batteries, catalytic and separation processes [21-25] are representative examples, notwithstanding the issues about toxicity. Indeed, it is well known that degradation of imidazolium cations determines the persistence of some cation fragments in the environment.[26] To decrease and eventually eliminate toxicity of aromatic imidazolium ILs is a current challenge. In this respect, Gathergood *et al*. have demonstrated that ether and ester groups on the side chain(s) of imidazolium-based ILs can reduce their bacterial toxicity,[27] as the ester bond represents a site of possible enzymatic cleavage.[12] Greener ILs have been also obtained using reagents derived from natural amino acids and organic acids.[28-29]

With this in mind, we synthesised new eco-friendly ILs bearing a motif derived from gluconic acid (Scheme 1). In all cases, the cation is indicated as $[\mathbf{N}_{xyzGAw}]^+$ where *x*, and *y* represent the length of alkyl groups depending on the diamine used, *z* is the number of the carbon atoms of the alkyl spacer of diamine, GA stands for gluconic amide and *w* represents the length of the alkyl chain used in the quaternization step.



Scheme 1. Structure of a) gluconic acid in equilibrium with glucono-δ-lactone; b) amide-based ILs; c) gluconate-based ILs.

Gluconic acid is a natural organic acid obtained from food waste same as rice, wine and vinegar.[30-31] Gluconic acid is obtained from the oxidation of glucose (Scheme 1a), and in nature, this chemical reaction is carried out by several bacteria and fungi.[32-34] This organic acid can be produced by chemical, electrochemical and biochemical ways.[35] Moreover, the use of gluconate in several medicaments and medical research as anion for salts like potassium, calcium, and iron gluconate led to the safe use of this molecule preserving human health.[36-38] Different studies had also demonstrated that this organic acid can be also involved in human metabolism.[39,40]

All above considerations led us supposing that the presence of such kind of residue on the cation or anion structure of ILs could allow obtaining salts of both low cyto- and ecotoxicity. Furthermore, the origin of gluconic acid and its wide availability make it cheap and its use as reagent is in the full respect of economical requirements of sustainability.

From a structural point of view, "sweet" ILs based on this molecule take advantage from the presence of the oligo-hydroxylated chain. The presence of several OH groups could play a dual function. Indeed, as above stated, it could contribute in decreasing IL toxicity, but it also endows the structure of high coordinating ability. Incorporating this feature, some of these ILs have been obtained[41-43] and in some cases, they have been applied to create ionic liquid gels and thermochromic systems aimed at environmental preservation.[44-45]

With respect to previous reports in literature,[41-43] our synthetic approach allowed inserting a gluconic-like residue also on the cation structure. Consequently, ILs in this work can be separated into two different classes, discerning gluconate- and gluconamide-based ILs.

Design of ILs structures was performed to evaluate the role played by the alkyl chain length and branching, the length of the spacer on the cation as well as the one deriving from the anion nature. Beside other factors, some previous reports have highlighted that branching the alkyl chain could induce a significant decrease in the ILs toxicity with respect the corresponding linear ones.[46]

ILs obtained were fully characterised, determining physico-chemical properties through TGA, DSC, viscosity and conductivity measurements.

Apart from considerations of a "greener" ionic liquid synthesis (with potential leakage of ILs), the cyto- and ecotoxicities were assessed.

In particular, three human cell lines, namely HeLa, HTC-116 and MCF-7 from cervical, colon and breast carcinoma were chosen as prototypes of human cells. Furthermore, haemolysis investigations were performed by using ovine blood cells.

As far as the aquatic toxicity is concerned, Zebrafish is considered as an ideal model for assessing the developmental toxicity of exposure to toxicants during early life stages and several studies were widely applied to test IL toxicity,[47] as a consequence of its transparency and rapid development of embryos. Moreover, Zebrafish shares 70% of genes with human genome and, if orthologous genes are considered, 47% of human genes have a one-to-one relationship with Zebrafish genes.[48,49]

In the light of all the above considerations, the main aim of this work was the obtainment of new ILs bearing on their structure moieties deriving from renewable sources and having both low cyto- and ecotoxicity. This should contribute to make more sustainable their whole life cycle.

The attention devoted to the synthetic procedure allowed identifying a protocol able to produce ILs in good yields minimizing the impact on the environment.

EXPERIMENTAL SECTION

Materials. Glucono-δ-lactone (Sigma Aldrich, ≥ 99.0 %), *N,N*-dimethylethylenediamine (TCI, > 98.0 %), *N,N*-dimethyl-1,3-propanediamine (Sigma Aldrich, 99.0 %), 1-(3-aminopropyl)imidazole (Sigma Aldrich, ≥ 97 %), 2-ethylhexyl bromide (Sigma Aldrich, 95.0 %), 1-bromobutane (Sigma Aldrich, 99.0 %), 1-bromooctane (Sigma Aldrich, 99 %), 1-bromododecane (Sigma Aldrich, 97 %), 1-iodobutane (Sigma Aldrich, 99 %), ethyl glycolate (Sigma Aldrich, 98 %), hexanoyl chloride (Sigma Aldrich, 97 %), *N,N,N′,N′*-tetramethylguanidine (Sigma Aldrich, 99 %), D-gluconic acid aqueous solution (Sigma Aldrich, 49-53 wt. % in H_2O) and lithium bis{(trifluoromethyl)sulfonyl}imide salt (TCI, > 98.0 %) were purchased and used without further purification. Dichloromethane (Merck, > 99.8 %), 1-butanol (Merck, > 99.8 %), methanol (Merck, HPLC grade), ethanol absolute (Merck, for analysis EMPARTA® ACS), acetonitrile (Merck, HPLC grade) and ethyl acetate (Merck, 98.8 %) were purchased and used without further purification.

Cell cultures. HeLa (human cervix adenocarcinoma), HCT-116 (human Colorectal Adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cell lines were cultured in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2, as previously described.[50-52] Cells having a narrow range of passage number were used for all experiments.

Cell viability assay. To test the cytotoxic effects of the ILs, the cells were plated at 5000 cells per well in 96-well plates and incubated for 24 h at 37 °C in a CO_2 incubator, as previously described.[52,53] ILs, diluted to the desiderated concentrations in culture medium, starting from 10^{-2} M solutions in water, were added to the wells for 24 h with respective vehicle control (H_2O). At the end of treatment, 20 μL of the Cell titer 96®AQ_{ueous} reagent was added to each well and incubated for 1–4 h at 37 °C in a CO_2 incubator. The absorbance was recorded at 490 nm using a 96-well plate reader (Sparks 20 M, Tecan Trading AG, Switzerland). The CellTiter 96® AQueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. The percentage of cell viability was calculated with respect to untreated control cells for each compound concentration after subtraction of the blank. The concentration necessary for 50 % of growth inhibition (IC_{50}) for each IL was calculated using a dose–response model, obtained from the sigmoidal fitting of response curves of percent inhibition versus logarithmic concentration of ILs using Graph Pad Prism software. Each result was the mean value obtained from experiments performed in triplicate.

Hemolytic activity assay. Hemolytic activity was evaluated with a blood agar medium supplemented with 5 % sheep blood. The blood agar was poured into 100 mm plastic plates. A tip was used to dispense 10 μL of each ILs (10^{-2} M concentrated). Plates were incubated for seven days at 37 °C. The test results were monitored and the presence of a distinctive translucent halo around the inoculum site was measured macroscopically.

Animals. Wild-type AB Zebrafish were obtained by European Zebrafish Research Center (Germany) and housed in ZebTEC semi-closed recirculation housing systems (Techniplast) at 28 °C, p*H* 7.5 and conductivity 500 µS on a 14/10 light/dark cycle. Fish were fed three times a day alternating dry food and brine shrimps. For the experiments Zebrafish fertilized eggs were collected in Petri dishes in fish water (1.2 mM $NaHCO_3$, 0.1 g/L instant ocean, 1.4 mM $CaSO_4$, methylene blue 0.00002 % w/v) at 28 °C and soon after transferred in 96 well plate, one embryo per well in 0.2 mL of embryo water. All the experiments were performed in agreement with EU Directive 2010/63/EU. The experimental protocol was approved by Italian Ministry of Health (Approval Animal Protocol No.1191/2016-PR).

Fish embryo toxicity test. Fertilized Zebrafish eggs were exposed to different concentrations of test chemicals compound for 96 h. Every 24 h, four observations were noted as indicators of lethality: the coagulation of fertilized eggs, the lack of somite formation, the lack of detachment of the tail-bud from the yolk sac, and the lack of heartbeat. At the end of the exposure period, toxicity was determined based on a positive outcome in any of the four apical observations recorded. Each experiment was done in triplicate using 20 embryos for group. Data are normalized to control lethality and expressed as mean ± standard error mean (SEM).

Exposure experiments. The chemical compound stocks were dissolved in physiological solution (pH =7.2) and kept at 4 °C. Working solution of 2 μM and 0.2 mM were prepared fresh daily in embryo water. Embryo without (control) or with the chemicals was changed daily.

Freshly fertilized zebrafish eggs were added to 0.2 mL of exposure medium per egg and incubated at 28 ± 1 °C for 24 h, 48 h, 72 and 96 h. For control, freshly fertilized zebrafish eggs were added to 0.2 mL of exposure medium without toxic compounds and incubated at 28 ± 1 °C for 24 h, 48 h, 72 h and 96 h. The exposure experiments were initiated as soon as possible after fertilization of the eggs. Daily observation was performed under stereomicroscope (Leica M165 FC).

NMR measurements. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker AV-300, Bruker ultrashield 400 plus, Bruker-spectroscopin 400 ultrashield and Ascend™ 600 Bruker nuclear magnetic resonance spectrometers. Chemical shifts were reported relative to $SiMe_4$.

Mass spectrometric measurements. ESI-MS mass spectrometric measurements were carried out on a Waters ICI Premier instrument with an Adylon Trìversa NanoMate injection system (cone voltage 50 V, source 120 °C). Both positive and negative ions were detected. For each sample, a methanol solution was prepared.

Thermogravimetric analysis (TGA). Thermogravimetric analysis was performed using a TGA/DSC thermogravimetric analyser from Mettler-Toledo, Inc. The samples were measured in alumina crucibles, at a heating rate of 5 K min^{-1}, under a dinitrogen atmosphere. The onset of the weight loss in each thermogram was used as a measure of the decomposition temperature (point at 5 % wt. loss of the sample).

Differential Scanning Calorimetry (DSC). DSC measurements were carried out using TA Instruments Modulated DSC Q 2000 V24.4 Build 116 with a refrigerated cooling system RCS 90, capable of controlling the temperature down to 220 K. Samples were weighed and hermetically sealed in aluminium pans. Heating and cooling rates were 10 °C min^{-1} for the ionic liquid sample. The maximum heating temperature was chosen depending on the thermal stability of the sample. The minimum cooling temperature was set at -50 °C under dinitrogen atmosphere. Two heating-cooling cycles were performed.

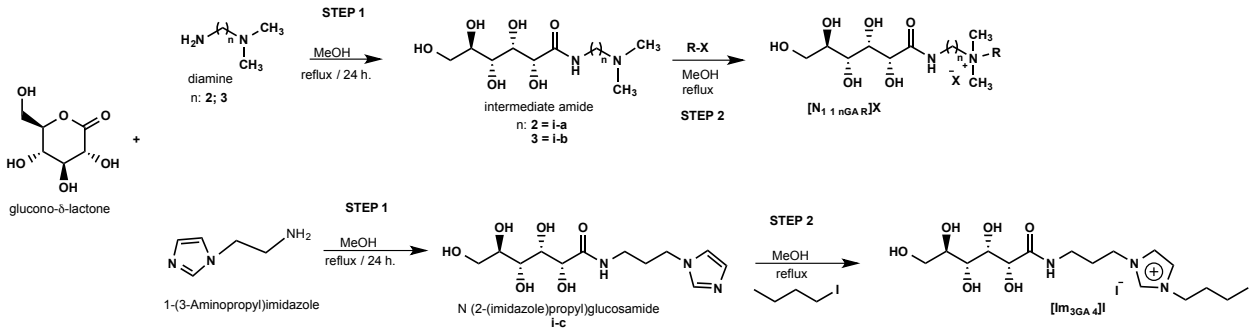
Viscosity measurements. Viscosity measurements were carried out using Marveln instrument Bohlin Gemini HR nano rheometer. Each sample was analysed in the range of temperature from 290 up to 375 K, with a rate of 2 K min^{-1}. The viscosity trends were fitted using the VFT model.

Conductivity measurements. The electrical conductivity σ (S/m) of the liquid salts was measured with a Keithley 2000 Multimeter in a two-points configuration on pure salts, at T = 297.15 K. Due to the small amount of sample to be analysed, a home-made cell with two microelectrodes was used; the cell constant was calculated using a commercial IL, $[C_8C_1Im][BF_4]$, whose conductivity is already reported in literature.[54] The conductivity values are the average of three measurements.

RESULTS AND DISCUSSION

Synthesis. As previously stated, two classes of "sweet" ILs were synthesized with the sugar moiety either present in the cation or the anion.

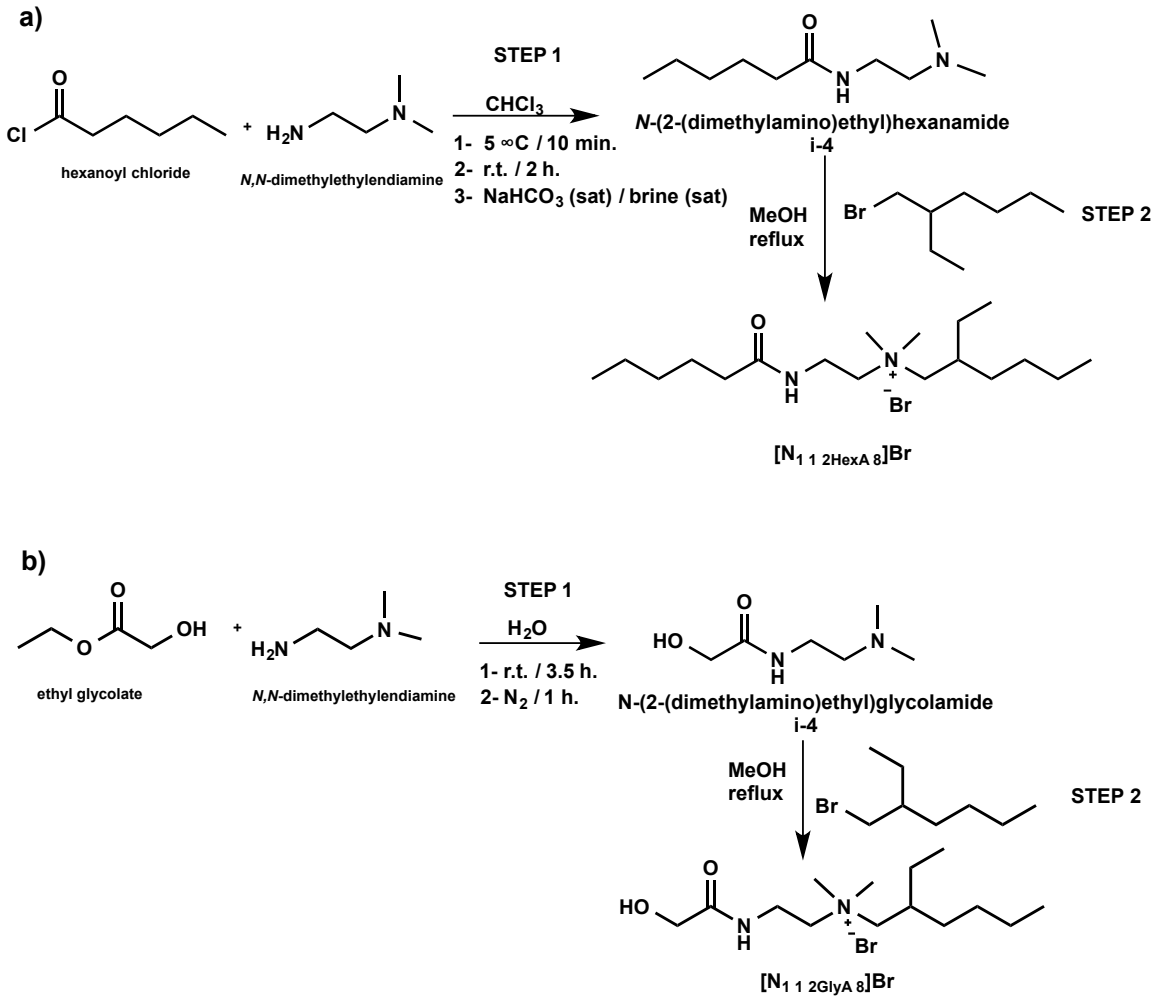
In order to introduce the sugar moiety in the cation, the 1-(3-aminopropyl)imidazole or aliphatic diamine was refluxed with glucono-δ-lactone, in MeOH solution for 24 h. In the second step, the amide intermediate was quaternised using different alkyl halides (Scheme 2).



Scheme 2. Synthetic pathways for glucono-based ILs.

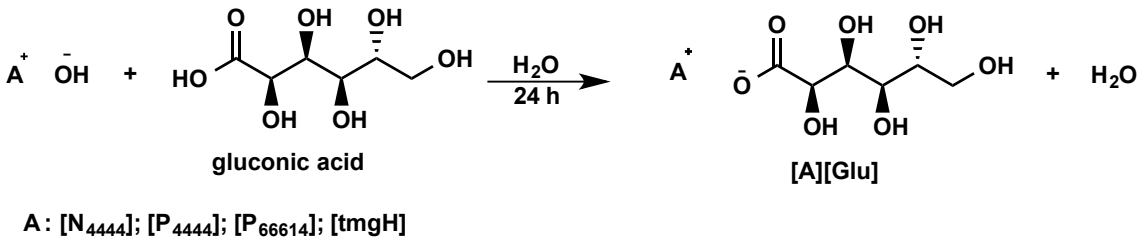
All the bis{(trifluoromethyl)sulfonyl}amide salts were prepared by the metathesis reaction with $LiNTf_2$, using 1-butanol as a solvent. The obtained ILs differ in terms of the length of the spacer between the amide and ammonium group, the alkyl chain length or branching and also the anion nature (Scheme 1).

As far as glycolic and hexanoyl derivatives (**$[N_{112}Gly_8]Br$** and **$[N_{112}HexA_8]Br$**) are concerned, the *N*,*N*-dimethylethylamine was reacted with ethylglycolate or hexanoyl chloride to obtain amide intermediates. These were successively quaternised by reaction with 2-ethylhexyl bromide to obtain the corresponding ammonium salts (Scheme 3).



Scheme 3. Synthetic pathways for the glycolic **(a)** and hexanoyl-based ILs **(b)**.

The synthesis of ionic liquids with the gluconate anion (Scheme 4) was described in our previous publication.[44] The ILs that we prepared differ in terms of the cation nature and, in some cases, the length of the alkyl chains (see Scheme 1).



Scheme 4. Synthetic pathways for the gluconate-based ILs.

It is worth noting the fact that synthetic protocols used to obtain "sweet" ILs respect to a good extent Green Chemistry Principles, according to the metric approach proposed by Clark *et al.* (Table S1).[55] Indeed, with only two exceptions related to the synthesis of **[$N_{112HexA8}$]Br** and **[N_{113GA8}][NTf_2]**, for which yields were lower than 70%, in all other cases they were higher than 89% (15 cases out of 22) or included in the range 70-89% (5 cases out of 22).

Atom economy (AE), with the only exception of the production of *N*-2-(dimethylamino)ethyl glucosamide and *N*-2-(dimethylamino)ethyl hexylamide, is always equal to 100% (Table S1). On the other hand, in most cases, reaction mass efficiency (RME) and optimum efficiency (OE) reach good levels as they change in the range 69.0-95.9%. Values failing the above range are the ones corresponding to the synthesis of $[NTf_2]^-$ ILs, **[$N_{112HexA8}$]Br** and for the synthesis of the above intermediates.

Mass Intensity (MI) values, as refereed to single step processes, fall in the range 1.19 - 49.85. The only black case is represented by the *N*-2-(dimethylamino)ethyl hexylamide. The lowest MI values were calculated for the synthesis of gluconate ILs.

Analysis of MIs calculated for gluconamide ILs shows as the workup of the product, consisting of a washing with ethyl acetate (30 mL), contributes to a large extent in determining the value (crf

columns 4 and 5 of Table S1). However, this contribution becomes less significant taking into consideration that a recommended and recyclable solvent was used.

As for the solvent nature, with the only exception of *N*-2-(dimethylamino)ethyl hexylamide, most of the reactions were performed in MeOH. *n*-BuOH was used for the anion exchange to obtain $[NTf_2]^-$ILs and in the case of gluconate-based ILs, water was used to carry out the anion exchange. Furthermore, if necessary, products were washed using ethyl acetate.

Energy considerations demonstrate how the reactions were performed under relatively mild conditions, as the highest temperature used was the one of the boiling point of MeOH.

In Vitro Cytotoxicity. All ILs were tested for their cytotoxicity. As above stated, three different human cancer cell lines were chosen as prototype of human cells, namely HeLa, HCT-116 and MCF-7, derived from cervical, colon and breast cancer lines respectively and previously used to test ILs cytotoxicity.[56-57-58] Cancer cells are generally more susceptible to toxicity than normal cells since they have a highly unstable genetic load and undergo rapid cell divisions.[59]

The cytotoxic effect of ILs was investigated by using the MTT assay.[60] The results, expressed as IC_{50} values, were calculated from the dose-response curves.

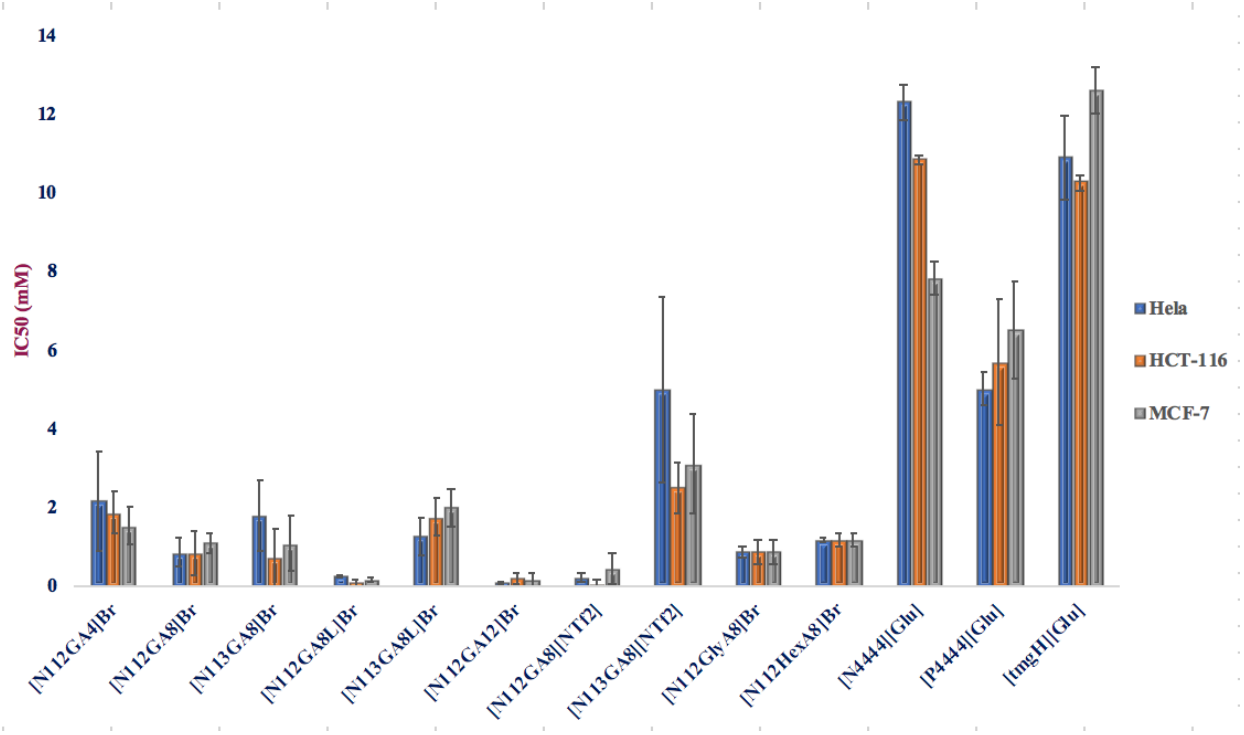


Figure 1. IC_{50} values as a function of the nature of ILs.

In the first set of experiments, carried out using stock solutions in water at 10^{-6} M, toxicity effect was recorded only for **[P_{66614}][Glu]** that prove to be the most toxic.

For this reason, the ILs toxicity was tested again by using more concentrated solutions (10^{-3} M) (Figure 1 and Table S2). Interestingly, despite the use of highly concentrated solutions, some ILs did not affect cell viability (see later).

In other cases, IC_{50} values range from 0.06 mM up to 12.6 mM. They proved to be quite high, with respect to the values generally reported in literature.[57,61]

In general, taking into consideration salts bearing the gluconate anion, IC_{50} values were significantly higher in the presence of ammonium or guanidinium cations than phosphonium one (Figure 1).

Besides, it is worthy of attention the increase in IC_{50} value due to the decrease of alkyl chain length on the phosphonium cation, going from **[P_{66614}][Glu]** to **[P_{4444}][Glu]**. Indeed, the latter exhibited toxicity effect only when a highly concentrated solution (10^{-3}M) was used. The above trend is in accordance with the literature.[62,63]

Conversely, all iodide-based ILs bearing a butyl chain and the gluconamide residue on the cation were found to be safe, regardless of the spacer length in (**[N_{112GA4}]I** and **[N_{113GA4}]I**) and cation nature (**[Im_{3GA4}]I** and **[N_{113GA4}]I**). The most surprising observation was the ultra-low toxicity of the imidazolium derivative, for which the presence of gluconamide moiety on the side chain appears to have removed toxicity effects completely. Indeed, toxicity effects were not detected also putting in contact cells with a 10^{-2} M solution of the salt. This result represents a significant improvement with respect the corresponding 1-hexyl-3-methylimidazolium halides.[64]

The results obtained by biological investigation on this component of new imidazolium class of compounds are quite interesting. The low toxicity due to the insertion of the gluconic moiety on the cation is in accordance with the literature, demonstrating the relevant role of the hydroxylated chain in in decreasing IL toxicity.[16]

According to literature,[18] ammonium salts bearing hydroxyl groups on the side chain proved less toxic than the corresponding alkyl derivatives, as accounted for by the comparison between **[$N_{112Gly8}$]Br** and **[$N_{112Hex8}$]Br**. However, IC_{50} value does not significantly change for glycolate and gluconamide salts, evidencing that the obtained result is not affected by the number of hydroxyl groups on the side chain.

Cytotoxicity also depends on the anion nature. Indeed, differently from **[N_{112GA4}]I**, toxicity effects were dectected for **[N_{112GA4}]Br** (Figure 1). Furthermore, analysis of data collected for 2-

ethylhexyl derivatives shed light on the role played by the length of the spacer. Indeed, both Br^- and $[NTf_2]^-$ ILs show a decrease in cytotoxicity from $[N_{112GA8}]^+$ to $[N_{113GA8}]^+$. Probably, the longer is the spacer, the higher is the conformational freedom of the cation that hampers the interaction with cell membrane. This hypothesis is well supported by data previously reported in literature about the increased biological activity of some bicyclic nucleosides with reduced conformational freedom in side chain.[65,66]

On the other hand, spacer being the same $[N_{112GAn}]^+$, cytotoxicity is also affected by the nature of the alkyl chain, linear or branched. In particular, linear alkyl chain results in a higher toxicity, as accounted for by the decrease in IC_{50} value from **$[N_{112GA8}]Br$** to **$[N_{112GA8L}]Br$** (Figure 1). However, the above effect depends on the spacer length being less significant in the case of the propyl spacer. Moreover, the comparison among IC_{50} values collected for **$[N_{112GA8L}]Br$** and **$[N_{112GA12L}]Br$,** evidences that the further lengthening of the alkyl chain, from octyl to dodecyl derivative, differently affects cytotoxicity in dependence of the different nature of the cell line (Figure 1; Table S2).

Hemolytic Activity. All ILs were also tested for their hemolytic activity. In this respect, it is well known that erythrocytes represent good systems to assess cytotoxicity of different molecules.[67] We used a blood agar test, in which 5% of sheep blood was included into an agar medium. ILs were tested at 10^{-2} M, and in major cases they did not show significant hemolytic activity (Figure 2).

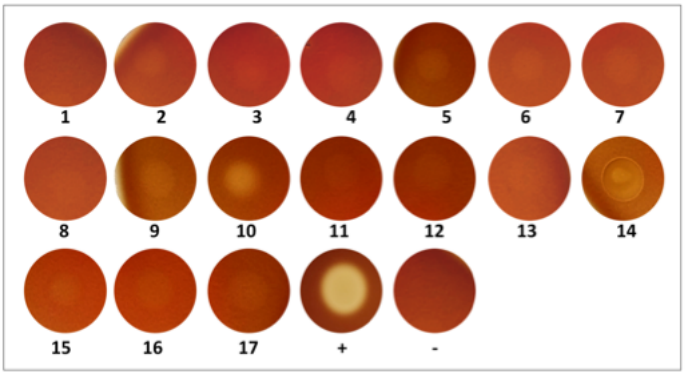


Figure 2. Blood agar hemolysis of ILs at 10^{-2} M, after 24h of incubation (1: **[N_{112GA8}]Br**; 2: **[N_{113GA8}]Br;** 3**: [N_{113GA4}]I;** 4: **[Im_{3GA4}]I;** 5: **[N_{112GA4}]I;** 6: **[N_{112GA8}][NTf_2];** 7: **[N_{113GA8}][NTf_2];** 8: **[$N_{112GA8L}$]Br;** 9: **[N_{113GA8}]Br;** 10: **[$N_{112GA12}$]Br;** 11: **[$N_{112Gly8}$]Br;** 12: **[N_{112GA4}]Br;** 13: **[$N_{112Hex8}$]Br;** 14: **[P_{66614}][Glu];** 15: **[P_{4444}][Glu];** 16: **[N_{4444}][Glu];** 17: **[tmgH][Glu]**). + and – represent positive and negative control, respectively.

A slight activity was detected in the case of **[P_{66614}]Glu**, according to the detected cytotoxicity and indicating that the antiproliferative effects are related to unspecific membrane damage. Less significant toxicity effects were detected for **[$N_{112GA12}$]Br** and **[$N_{113GA8L}$]Br**.

Fish embryo toxicity test. Toxicity of ILs was evaluated in Zebrafish from 24 to 96 hours post fertilization (hfp).[68] The coagulation of fertilized eggs, the lack of heartbeat, the lack of detachment of the tail-bud from the yolk sac and the lack of somite formation were daily checked. In this case, based on the results collected through cytotoxicity tests, we selected only some gluconamide-based ILs to assess the effect of the spacer, anion and alkyl chain length. Furthermore, we tested ILs bearing the gluconate anion and the hexylamide derivative, **[$N_{112Hex8}$]Br**, to evaluate the relevance of the presence of hydroxyl groups on the side chain. All the ILs were tested at 2 μM and 0.2 mM. The above concentrations were chosen on the basis of previous reports in literature.[63,69] No

significant toxicity was detected at both concentrations, as we didn't observe significant differences in treated and untreated fish (Figure 3 and Table S3).

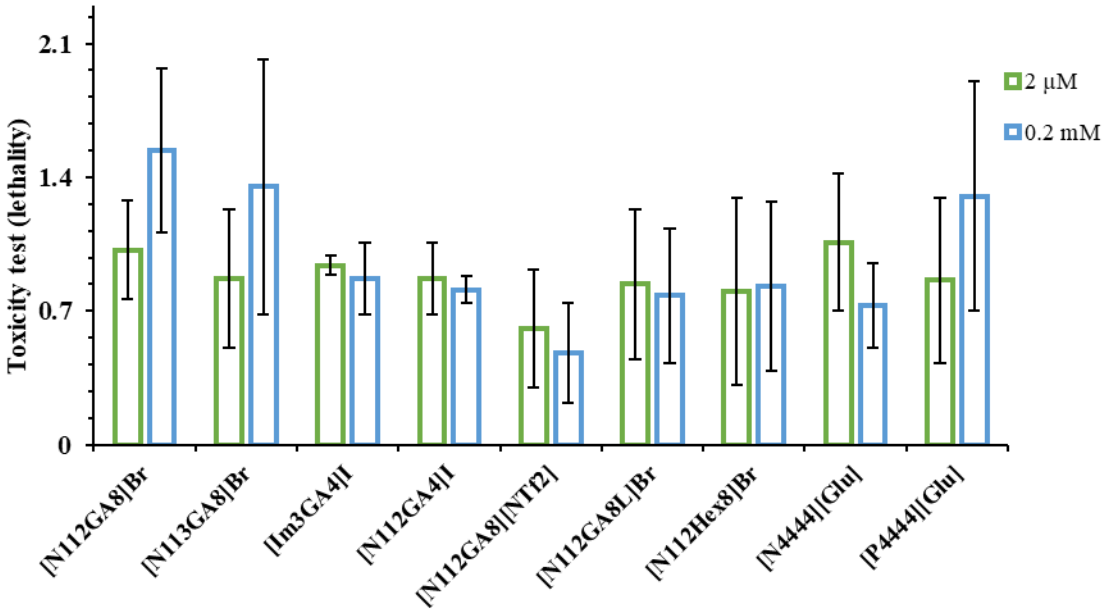


Figure 3. Results of toxicity test performed on Fish embryo (lethality at 24/48 h).

We did not detect toxicity effect also for imidazolium derivative, indicating that the presence of the oligo-hydroxy motif is able to compensate the negative effect of the imidazolium cation.

Physical properties of 'sweet appended' ionic liquids

TGA investigation. Thermal stability of ILs was determined using TGA measurements (Figure S1 and Table S4 of SI). In all cases, the temperature corresponding to the 5% of weight loss was considered (T_d).

In the case of gluconoamide-based ILs, T_d ranges from 127.8 up to 210.3 °C. For the sake of clarity, data collected will be discussed as a function of the changing structural factors. (Figure 4).

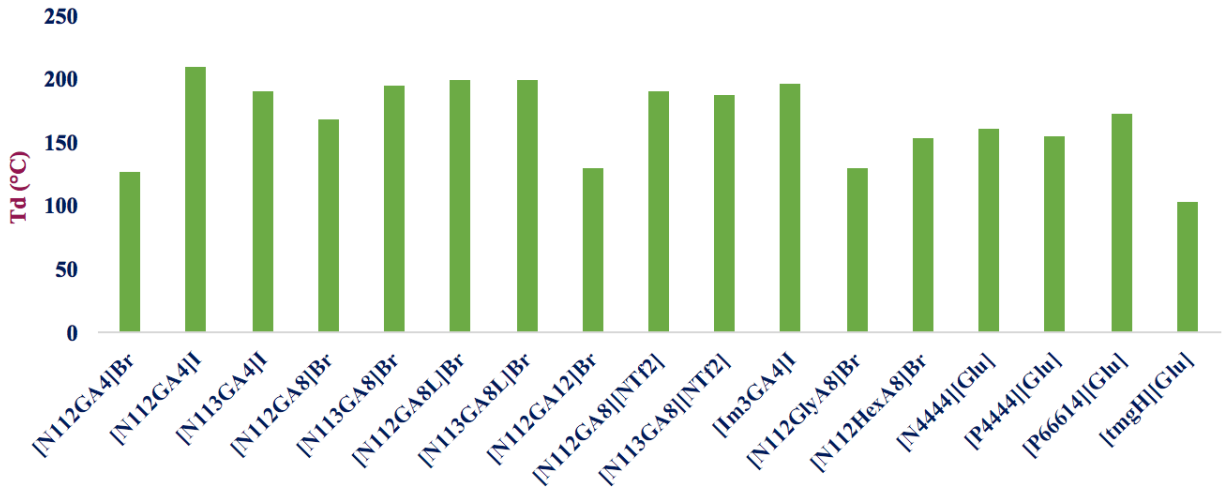


Figure 4. Trend of T_d (°C) values as a function of IL nature.

As far as the length of the alkyl spacer is considered, the trend of T_d values depends on the anion nature and the length of the alkyl chain on the ammonium head. Indeed, for the 2-ammonium derivatives (**[N_{112GA8}]Br**, **[N_{113GA8}]Br, [N_{112GA4}]I, [N_{113GA4}]I, [N_{112GA8}][NTf_2]** and **[N_{112GA8}][NTf_2]**), the lengthening of the alkyl spacer induces an increase in T_d values for Br^- – ILs and a decrease in the case of I – ILs and $[NTf_2]^-$ ILs, with more significant changes in the presence of halide anions with respect to $[NTf_2]^-$ anion (ΔT = 26.7, 18.6 and 2.6 °C for Br^-, I^- and $[NTf_2]^-$ ILs, respectively). The effect of the anion nature on the thermal stability of ILs is well documented in literature.[70-71]Furthermore, in our case, the comparison between T_d values corresponding to **[N_{112GA4}]Br** and **[N_{112GA4}]I** allows stating that thermal stability increases decreasing the anion nucleophilicity, according to the data previously reported for imidazolium salts.[72]

Regardless of the spacer length, the branching of the alkyl chain induces a decrease in thermal stability (**[N_{112GA8}]Br** and **[$N_{112GA8L}$]Br**, **[N_{113GA8}]Br** and **[$N_{113GA8L}$]Br;** ΔT **=** 32.2 and 5.5 °C for **[N_{112GAn}]$^+$** and **[N_{113GAn}]$^+$** ILs, respectively).

On the other hand, as far the effect of the alkyl chain length on the ammonium head is concerned, the data collected allow identifying the linear octyl chain as the superior feature. Indeed, T_d values change along the following trend: **[N_{112GA4}]Br < [$N_{112GA8L}$]Br > [$N_{112GA12}$]Br**.

Finally, the comparison between T_d values collected for **[Im_{3GA4}]I** ad **[N_{113GA4}]I** indicates higher thermal stability of the imidazolium salt with respect to the corresponding ammonium one, according to what was previously observed by Afonso *et al*., comparing thermal properties of $[N_{1888}]^+$ and $[C_8C_1im]^+$ ILs.[73]

As stated above, to evaluate the effect of the presence of oligo-hydroxylated chain, glycolic and hexyl derivatives, **[$N_{112GlyA8}$]Br** and **[$N_{112HexA8}$]Br,** were also synthesized. In both cases, the comparison of T_d values with the one corresponding to **[N_{112GA8}]Br** evidences how the presence of a "sweet" chain represents a warranty of higher thermal stability.

As for gluconate-based ILs, T_d ranges from 103.1 up to 172.5 °C. Among the analysed salts, **[tmgH][Glu]** showed the lowest thermal stability, whereas the **[P_{66614}][Glu]** was the most thermally stable. In general, T_d values depend on the cation nature, with ammonium IL exhibiting a higher thermal stability with respect to the corresponding phosphonium one (**[N_{4444}][Glu]** and **[P_{4444}][Glu]**). Finally, for phosphonium ILs, the elongation of the alkyl chain induces a corresponding increase in T_d (**[P_{4444}][Glu] and [P_{66614}][Glu]**), according to the previous reports.[70]

DSC investigation. Solid-liquid transition phase of the new ILs was investigated by performing DSC measurements. In general, they showed glass transitions (T_g) detected in the heating cycle and displayed in Figure 5 (see also Table S4 and Figure S2).

According to the previous reports, this behavior indicates that they have a weak tendency to crystallize.[74-75]

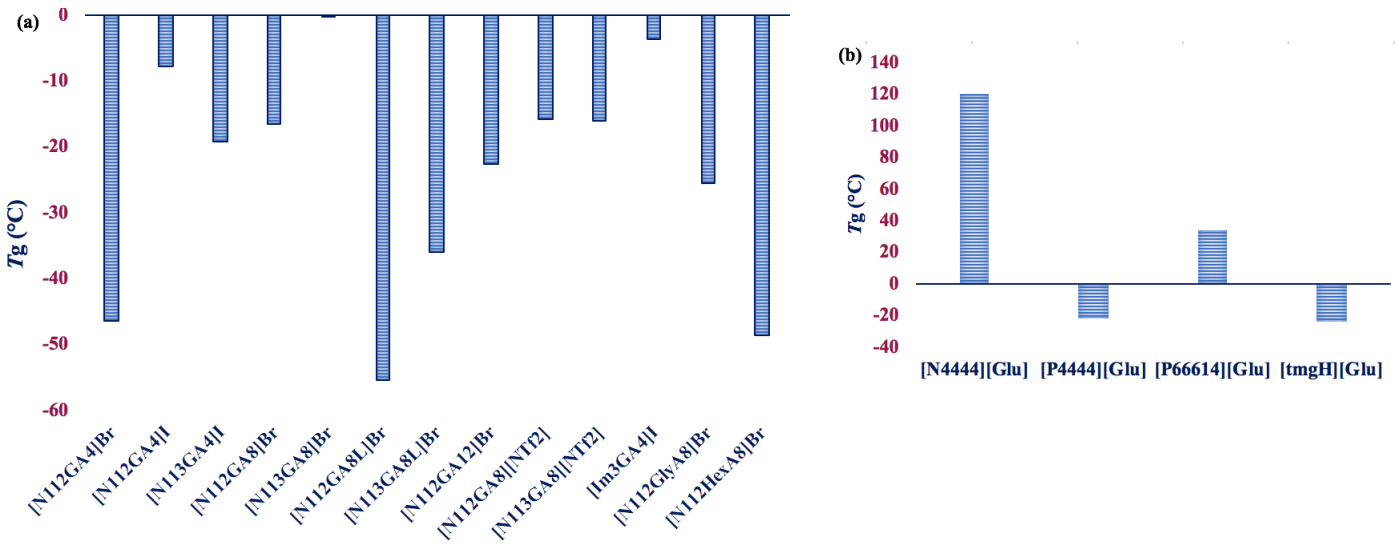


Figure 5. (**a**) Glass transition temperatures (T_g) for amide-based ILs; (**b**) Glass transition temperatures (T_g) for gluconate-based ILs.

With the only exception of **[N_{4444}][Glu]**, T_g values lower than 100 °C were detected, allowing to classify almost all salts as ILs. In the case of gluconamide-based ILs, they range from -55.5 (**[$N_{112GA8L}$]Br**) up to -0.1 °C (**[N_{113GA8}]Br**).

For the above ILs, T_g values are affected by both the anion nature and alkyl chain length. In the case of butyl derivatives, they increase from **[N_{112GA4}]Br** to **[N_{112GA4}]I** and, taking into consideration the iodide salts, they also decrease by lengthening the alkyl spacer between the amide and ammonium group (**[N_{112GA4}]I** and **[N_{113GA4}]I**).

Spacer being the same, the lengthening of the alkyl chain on the ammonium head induces firstly a decrease and then an increase in T_g values, as accounted for by the comparison amongst the data collected for **[N_{112GA4}]Br**, **[$N_{112GA8L}$]Br** and **[$N_{112GA12}$]Br**. This trend perfectly agrees with the one generally detected for alkylimidazolium salts that shows a decrease in melting temperature until conformational freedom of the alkyl chain prevails over van der Waals interactions.[76]

Among structural factors, also the branching of the alkyl chain plays a role. Indeed, irrespective of the spacer nature, salts bearing the linear alkyl chain exhibit T_g values lower than the one bearing the 2-ethylhexyl chain.

A more complex trend was detected as far as the effect of the length of the spacer is concerned. Indeed, in the case of bromide derivatives, T_g increases from **[N_{112GAn}]$^+$** to **[N_{113GAn}]$^+$** ILs. No significant changes were detected for $[NTf_2]^-$ ILs, while a decrease in T_g values was collected from **[N_{112GA4}]I** to **[N_{113GA4}]I**.

Comparison among data collected for **[N_{112GA8}]Br**, **[$N_{112GlyA8}$]Br** and **[$N_{112HexA8}$]Br** accounts for a decrease in T_g values in parallel with the decrease in the number of hydroxy groups featuring the amide chain. Finally, T_g values also decrease moving from imidazolium to ammonium salt (**[Im_{3GA4}]I** and **[N_{113GA4}]I**).

In general, gluconate-based salts show higher T_g values with respect to gluconamide-based salts. Indeed, in this case, they range from -24.1 (**[tmgH][Glu]**) to 120 °C (**[N_{4444}][Glu]**).

[P_{4444}]Glu] shows two glass transitions, the first one occurring at -21.8 °C and the second one at 80.2 °C. For these salts, changes in the cation nature significantly affect T_g values, as accounted for by the comparison between **[N_{4444}][Glu]** and **[P_{4444}][Glu]**. On the other hand, for phosphonium

salts, a significant increase in T_g was also detected as a consequence of the alkyl chain lengthening for (**[P_{4444}][Glu]** and **[P_{66614}][Glu]**).

Viscosity measurements. Some gluconamide ILs were sufficiently fluid at room temperature to allow their viscosity to be determined using a rheometer equipped with a thermostat. Viscosity values as a function of the IL nature are displayed in Figure 6 (Table S5).

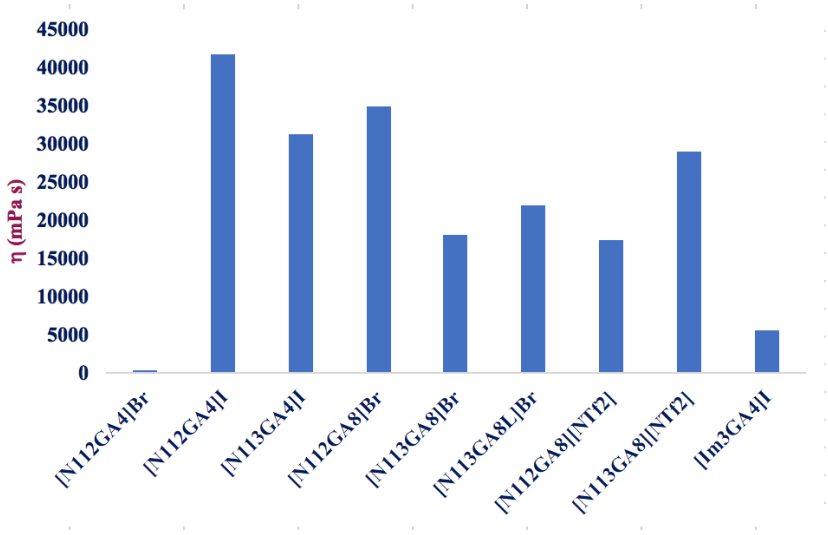


Figure 6. Viscosity (η / mPa s) values for gluconamide-based ILs.

η values range from 106.3 mPa s up to 41700 mPa s, indicating **[N_{112GA4}]Br** and **[N_{112GA4}]I** as the ILs showing the lowest and the highest viscosities, respectively. The above observation allows stating that, in the case of halide based ILs, η depends on the ion size and polarizability, according to previous reports in literature.[77]

As far as the length of the alkyl spacer is concerned, for halide-based ILs (**[N_{112GA4}]I**, **[N_{113GA4}]I**, **[N_{112GA8}]Br** and **[N_{113GA8}]Br**), η values decrease with the increase in the length of the alkyl spacer.

Surprisingly, in the case of $[NTf_2]^-$ ILs (**[N_{112GA8}][NTf_2]** and **[N_{113GA8}][NTf_2]**) an opposite trend was observed.

The nature of the alkyl chains on the ammonium head also affects viscosity, as accounted for by the decrease in η values on going from **[N_{113GA8}]Br** to **[$N_{113GA8L}$]Br**, indicating that the branching of the alkyl chain gives rise to less viscous solvents.[32]

Finally, alkyl chain length and anion being the same, η values proved lower for imidazolium than for ammonium-based ILs, as accounted for by the data collected for **[N_{113GA4}]I** and **[Im_{3GA4}]I**, in perfect agreement with the data previously reported in literature.[78]

Viscosity values were also measured as a function of the temperature (from 300 up to 370 K) and the trends observed were analysed with the VFT (Vogel-Fulcher-Tamman) model (Figure S3). The model is represented by the eq. (1)[79]

$$\log \eta = A + \frac{D \cdot T0}{(T - T0)} \qquad (1)$$

where A accounts for the viscosity at the infinite temperature limit, T0 is the temperature at the infinite viscosity and D represents the fragility that measures the strength for glass forming materials.[80]

In general, in the case of fragile glass materials featured by the occurrence of feeble interactions and hydrogen bonds, D values are lower than 10.

Interestingly, D values are related to the fragility index, *m*, by the eq. (2):[81]

$$m = 16 + \frac{590}{D} \qquad (2)$$

This parameter is related to the structural stability of the materials to the thermal degradation. For glass-forming materials, *m* values are higher than 100.

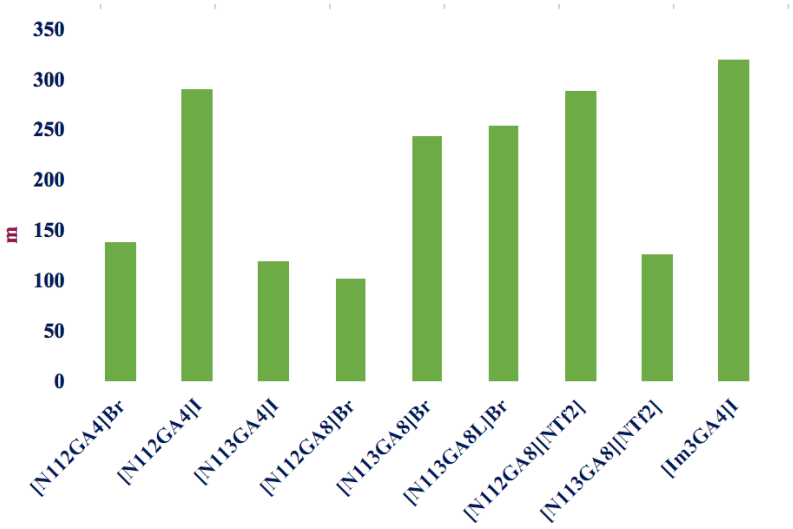


Figure 7. Fragility index (*m*) for gluconamide-based ILs.

m values obtained for gluconammide-based ILs are displayed in Figure 7 (Table S5 and Figure S3). Analysis of both D and *m* values account for the glass material nature of our ILs. Indeed, D range from 1.9 (**[Im$_{3GA4}$]I**) up to 6.8 (**[N$_{112GA8}$]Br**). Conversely, *m* values range from 102.3 (**[N$_{112GA8}$]Br**) and 319.7 (**[Im$_{3GA4}$]I**).

Both parameters significantly change as a function of all the structural features considered so far. In particular, for butyl derivatives, *m* values decrease as a function of the anion nature according to the increase in size and polarizability of the anion (**[N$_{112GA4}$]Br > [N$_{112GA4}$]I**).

It is worth of noting the fact that, as far as the alkyl spacer is concerned, trend observed is also significantly affected by the length of the alkyl chain on the ammonium head and the anion nature. Indeed, in the case of butyl derivatives (**[N_{112GA4}]I** and **[N_{113GA4}]I**) and for 2-ethyl-hexyl derivatives associating with the $[NTf_2]^-$ anion (**[N_{112GA8}][NTf_2]** and **[N_{113GA8}][NTf_2]**), *m* values increase on going from the ethylene to the propylene spacer. Conversely, for **[N_{112GA8}]Br** and **[N_{113GA8}]Br**, an opposite trend was detected.

The nature of the alkyl chain, linear or branched, affects to a lower extent *m* values as accounted for by the comparison between **[N_{113GA8}]Br** and **[$N_{113GA8L}$]Br**. Finally, the nature of the cation affects fragility index values as testified by the significant decrease in *m* values, going from **[N_{113GA4}]I** to **[Im_{3GA4}]I**.

Conductivity measurements. Conductivity measurements were carried out for most gluconamide ILs, to gain insights into the effects that structural changes may have on their ability to act as charge carriers. σ (S/m) values, measured using **[C_1C_8Im][BF_4]** as standard, are displayed in Figure 8 (Table S6 of SI).

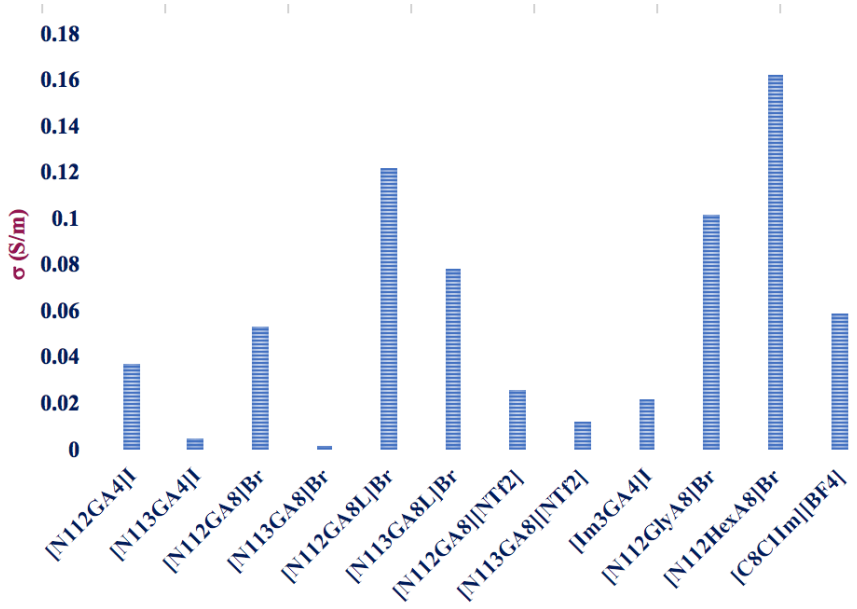


Figure 8. Conductivity (σ) values for some of ILs obtained.

The collected σ values range from 0.002 up to 0.162 S/m. Analysis of the above values shows that one of the main factors determining conductivity of our ILs is represented by the nature of the alkyl linker, as accounted for by its decrease going from ethyl to propyl spacer. Interestingly, the degree of decrease depends on the anion nature, more significantly with the increase in the anion coordination ability ($Br^- > I^- > [NTf_2]^-$).

With anion being the same, conductivity significantly increases from $\mathbf{[N_{11nGA8}]^+}$ to $\mathbf{[N_{11nGA8L}]^+}$ ILs, according to the decrease in the branching of the alkyl chain. On the contrary, the ability of charge transport increases from $\mathbf{[N_{112GA8}]Br}$ to $\mathbf{[N_{112HexA8}]Br}$, where OH groups on the side chain range from 5 to none. The former, favoring the formation of hydrogen bonds, induces a parallel increase of the ILs viscosity and a corresponding decrease in σ values.

CONCLUSIONS

Taking into consideration the sustainability endorsement and the incessant request for the identification of new solvents potentially applicable in the industry with low environmental impact, we have presented, here, a new class of organic salts bearing in the structure a residue derived from gluconic acid. Since gluconic acid can be harnessed from food waste, its utilization to obtain non-toxic solvents from renewable sources is highly desirable from a 'green chemistry' point of view.

In particular, we prepared aliphatic organic salts, namely ammonium and phosphonium ones, bearing the "sweet" residue on the cation or anion. The synthetic procedures used comply, to a good extent, with Green Chemistry metrics approach and principles.

Analysis of solid-liquid transition and thermal stability allowed stating that the majority of these salts fall in the class of ILs that are suitable solvents for applications at high temperature.

As expected, their physico-chemical properties are significantly affected by changes in the structure. Toxicity of ILs obtained was assessed towards three different cell cancer lines and fish embryos. To our surprise, we demonstrated that they are able to exert moderate or low anti-proliferative effects and prove safe also towards human erythrocytes. Toxicity effects were detected only using unusual highly concentrated solutions ($IC_{50} \sim 10^{-3}$ M), and they change in dependence of the presence of the gluconic moiety on the cation or on the anion. In general, lower IC_{50} values were detected for amide based ILs. Furthermore, as far as ecotoxicity studies are concerned, "sweet" ILs proved completely safe towards to Zebrafish embryos, as no lethality events were observed at 24 or 48 hours.

It is noteworthy, that the above considerations can be also drawn for the imidazolium derivative. The above result testifies that the presence of a "sweet" residue, such as the one derived from gluconic acid, could pave the way to the production of safer aromatic ILs, with favorable consequences from an industrial point of view.

ASSOCIATED CONTENT

Supporting Information. Synthetic details; DSC and TGA traces, VFT traces, Table of IC_{50} values, Table of toxicity test performed in the presence of zebrafish embrios, Table of DSC and TGA data, Table of viscosity data, Table of conductivity data, ^{1}H and ^{13}C NMR spectra.

The following files are available free of charge.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Declaration of interest

The authors declare no competing financial interest

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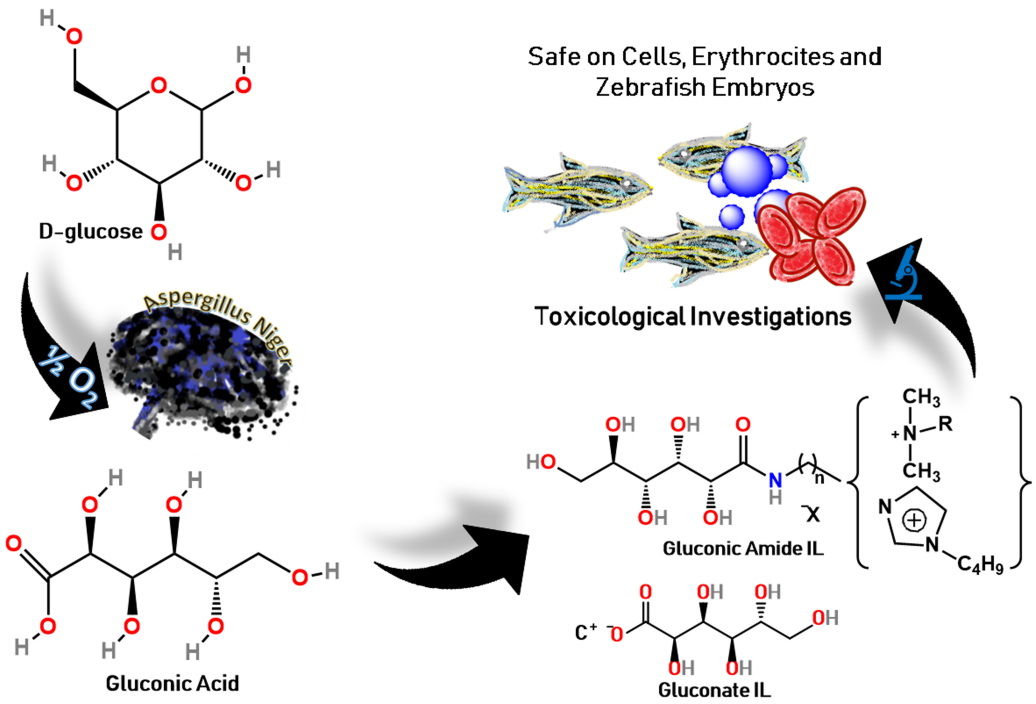

SYNOPSIS. “Sweet” ionic liquids incorporating moiety deriving from food waste were obtained. They show good physico-chemical properties and drastically reduced toxicity.