Title: Comparison of acidic deep eutectic solvents in production of chitin nanocrystals

Corresponding Author: Dr. Hailan Lian, Ph.D.

Corresponding Author's Institution: Nanjing Forestry University

First Author: Yang Yuan

Order of Authors: Yang Yuan; Shu Hong; Hailan Lian, Ph.D.; Kaitao Zhang; Henrikki Liimatainen

Abstract: Five different acidic deep eutectic solvents (DESs) composed of choline chloride and organic acids were applied to fabricate chitin nanocrystals (ChNCs). All DESs resulted in high transmittance and stable ChNCs suspensions with very high mass yield ranging from 78% to 87.5% under proper reaction conditions. The acidic DESs had a dual role in ChNCs fabrication, i.e. they promoted hydrolysis of chitin and acted as an acylation reagent. Physicochemical characterization of chitin revealed that the removal of amorphous area during DES treatments led to increased crystallinity of ChNCs and a dimension diversity correlated the DES used. The average diameter and length of individual ChNCs ranged from 42 nm to 49 nm and from 257 nm to 670 nm, respectively. The thermal stability of ChNCs was comparable to that of pristine chitin. Thus, acidic DESs showed to be non-toxic and environmentally benign solvents for production of functionalized chitin nanocrystals.

Dear Editor in Chief,

Thank you for your and reviewers' comments concerning our manuscript entitled "Comparison of acidic deep eutectic solvents in production of chitin nanocrystals" (CARBPOL-D-19-04245). We have revised our manuscript accordingly. Please see it in our revised manuscript highlighted with red. The responses to Reviewers' were listed point-by-point in response to reviewers.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly and hope that our responses are adequate enough. Once again, thank you very much for your comments and suggestions.

Yours sincerely,

Hailan Lian

lianhailan@njfu.edu.cn

Highlights:

1. Efficient hydrolysis of chitin in organic acid deep eutectic solvent (DES).

2. Physicochemical properties of ChNCs were compared prepared with different acidic DESs.

3. Choline chloride and lactic acid DES is optimal choice in making lactate ChNCs.

4. Green and fast production of ChNCs in lactic acid DES with yield up to 87.5%.

Paper Title: Comparison of acidic deep eutectic solvents in production of chitin nanocrystals
Ms. No.: CARBPOL-D-19-04245

Dear Editor-in-Chief,

We would like to thank you and all reviewers for the constructive feedback and great advices given in the comments. We have done our best to make the required corrections and truly hope our responses are adequate enough.

Below are all the comments with our answers. Responses to reviewers' comments are shown in blue. Feel free to contact us if there is any additional requests or comments concerning the manuscript.

Comments:

Reviewer #1: Reference paper: CARBPOL-D-19-04245
1- While the English grammar is acceptable, a thorough read through would improve the paper.
Examples:
*Lines 15: reformulate
Response: The manuscript was checked by the commercial proof-reading service (Scribendi). In addition, we have reformulated some sentences. Please see it in the revised version.

*Line 71: remove "were used"
Response: We rewrote this sentence as 'The morphology of the synthesized ChNCs was observed through atomic force microscope (AFM). The physicochemical characteristics of ChNCs were studied by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and thermogravimetric analysis (TGA).'

*Line 128: use "heating rate" instead of "temperature gradient"
Response: We have revised it as "heating rate".

*Line 219: use "penetrates" instead of "penetrate"
Response: We revised it as "penetrates ".

*Line 220: use "promotes" instead of "promote"
Response: We revised it as "promotes ".

*Line 240: use "examine the effect" instead of "examine effect"
Response: We revised it as "examine the effect ".

2-The performed work is very interesting. Consequently, highlights need to be improved focusing on the main new results.
Response: We have rewritten the highlights according to the main results.

3-How do you control the high hygroscopicity of choline chloride during the preparation of the DESs?

Response: Choline chloride was first dried in an oven in a conical flask, the weighted other components were added the flask quickly. Moreover, the water associated with hygroscopicity of DES did not affect the hydrolysis reaction of chitin as proved by previous study, in which hydrated DES were used (Ma Y, et al. ACS Omega, 2019, 4(5): 8539-8547; Sirviö J A et al. Biomacromolecules, 2016, 17(9): 3025-3032.).

Ma Y, Xia Q, Liu Y, et al. Production of Nanocellulose Using Hydrated Deep Eutectic Solvent Combined with Ultrasonic Treatment[J]. ACS Omega, 2019, 4(5): 8539-8547

Sirviö J A, Visanko M, Liimatainen H. Acidic deep eutectic solvents as hydrolytic media for cellulose nanocrystal production[J]. Biomacromolecules, 2016, 17(9): 3025-3032.

4-The authors should compare the obtained results with some results already reported for the production of nanochitin using DES.

Response: We compared the prepared nanochitin with those previously reported. Please see it in section of 'Morphology of ChNCs'

Reviewer #2:

1. Rewrite and shorten the conclusion.

Response: We have rewritten the conclusion part.

2. Five kinds of acidic DESs were studied in this work. Please clarify the optimal solvent for preparing ChNCs based on the discussion.

Response: Among them, DES of choline chloride and lactic acid was considered as the optimal choice due to the short reaction time, the highest mass yield of ChNCs (up to 87.5 wt-%), and stability of aqueous ChNC dispersion. This point was clarified in the conclusion part.

3. The mean sizes of ChNCs obtained from five DESs were different with each other. From the AFM images, individuals and aggregates coexisted in all of suspensions. How to avoid the aggregation of ChNCs?

Response: The ChNCs were dispersed in neutral conditions and then dried on mica sheets for observation the morphology. During the drying procedure the diluted ChNCs may aggregate and form the bundles, it is hard to avoid that. To observe individual ChNCs, the suspensions should be diluted to a lower consistency. Besides, the pH of the suspension is also a key factor to avoid the aggregation of ChNCs. Therefore, we belive that the aggregation is partly because of AFM sample preparation, and may also be caused by inherent existence of bundles in the ChNC dispersion. (Qi Z D et al. RSC Advances, 2013, 3(8): 2613-2619; Jiang J et al. Journal of agricultural and food chemistry, 2018, 66(43): 11372-11379.).

Qi Z D, Fan Y, Saito T, et al. Improvement of nanofibrillation efficiency of α-chitin in water by selecting acid used for surface cationisation[J]. RSC Advances, 2013, 3(8): 2613-2619.

Jiang J, Yu J, Liu L, et al. Preparation and Hydrogel Properties of pH-Sensitive Amphoteric Chitin Nanocrystals[J]. Journal of agricultural and food chemistry, 2018, 66(43): 11372-11379.

4. What about to control the size of ChNCs by using the same DES solvent?

Response: This study focused on comparison of production of ChNCs with different acidic DESs.
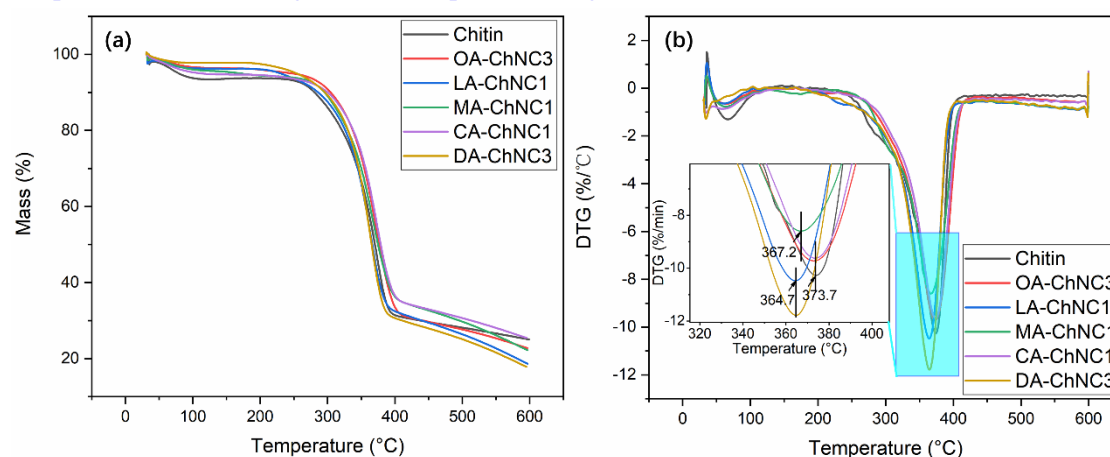
The morphology of obtained ChNCs is likely affected by the pKa value of DES and possible acylation reactions. For the specific DES, the size of ChNCs can be controlled by changing the reaction conditions, i.e. reaction time and temperature, or ultrasonication procedure. However, we did not address this issue further in the present study, despite it is an interesting and important topic.

5. It's better to provide the discussion about the benefits or weak of ChNCs prepared by using acidic DESs and other approaches (Tempo-strategies, etc.).

Response: We included related discussion in the section 'Optical properties and mass yield of ChNCs suspensions' to show the benefits of acidic DESs compared to HCl method. Moreover, we compared the DESs used here for production of ChNCs with previous reported method based on DES in the section 'Morphology of ChNCs'.

6. Please improve the quality of Figure 5.

Response: We have enlarged the insert picture in Figure 5b.

7. Developing the novel solvents for fabricating chitin including solutions or nanoparticles is a significant research for exploring functional materials based on chitin. It's better to emphasize the importance of this research in the Introduction part. The related literature can be cited. (Carbohydrate Polymers, 177 (2017), 297-305.)

Response: Thank you for your suggestion. We have added related content in introduction part. Please see it in the revised version.

Reviewer #3: Recommendation

Major revision

Manuscript details

Manuscript Number: CARBPOL-D-19-04245

Title: Comparison of acidic deep eutectic solvents in production of chitin nanocrystals

Yuan, Hong, Lian, Zhang, Liimatainen

Overview and general recommendations

The manuscript is devoted to the utilization of different acidic DESs for the production of chitin nanocrystals from commercial chitin. The results presented by the authors indicate that with their proposed method good yields and high cristallinity of the final material can be achieved. The authors suggest the DES acted also as grafting agent, improving the crystals dispersibility.

The present manuscript presents methodology and results worth publishing on Carbohydrate Polymers. However, there are some issues the authors must address. Mainly, authors should provide additional proof of the esterification of chitin units with organic acids. Secondly, the scientific style of the manuscript needs to be reviewed in multiple passages. The research on chitin nanocrystal is not adequately introduced and put into context. Additional discussion of the provided data is also required. Authors should check journal guidelines regarding references.

Response: We have revised the manuscript accordingly and included the research related to chitin nanocrystals in the introduction part and additional discussion related to the data. Please see it in the revised version.

Comments

Highlights

Highlight number 3 should be reviewed in style, since there is no connection between thermal stability and yield.

Response: We have rewritten this part.

Abstract

At line 9, authors indicate all nanocrystal suspensions as "transparent". This is not true, since some suspensions have shown poor transmittance. At line 14, authors state "morphological diversity [of chitin NCs] correlated the DES used". In which sense there is correlation? The style of conclusion of the abstract (lines 17-18) should be reviewed.

Response: We have revised this statement as 'All DESs resulted in high transmittance and stable ChNCs suspensions with very high mass yield ranging from 78% to 87.5% under proper reaction conditions.'

The dimension of ChNCs is related to DES used. We have revised morphological diversity to 'dimension diversity'.

In abstract, we have revised the conclusion as 'Thus, acidic DESs showed to be non-toxic and environmentally benign solvents for production of functionalized chitin nanocrystals.'.

Introduction

The introduction should be reviewed in style in multiple passages (lines 24, 26, 28, 29, 38, 48, 62, 64, 65). It is not clear, at lines 38-40, whether TEMPO and alkali can be used to produce

nanocrystals. Lines 42-43 describes acidic conditions, with no clear connection to the previous or following lines. The purpose of Lines 43-44 is unclear as well (is esterification involved in the production of nanocrystals?). The importance of production of chitin nanocrystals is not clear to the reader. Also, is ultrasonication commonly used to produce chitin nanocrystals? Before or after acid/alkali treatment?

Response: We have revised and rewritten the related citations in the introduction section. TEMPO can be used to prepare ChNCs, we have added this statement in this sentence.

The content in Lines 42-44 introduced the main methods of production of well dispersed ChNCs. We have rewritten this part and made it better connected with the whole content

.

Ultrasonication is commonly used to produce chitin nanocrystals after acid/alkali treatment.

Materials and methods

Are degree of acetylation and protein content of starting chitin known to the authors?

Response: The chitin source was crab shell. The degree of acetylation was 95% as determined through titration method. The protein content in chitin was 0.99% as measured by Bradford method.

The style of line 93 should be revised. Was the washing water deionized?

Response: Yes, it was deionized water. This was described in the 'materials and method' as 'Deionized water was used throughout the whole experiment.'

Table 1. I suggest to include in this table the different nanocrystals obtained, as a quicker reference, because the acronyms used are long and not immediate.

Response: Thank you for your suggestion. We prefer keeping it in the current form, since this part addresses the preparation of DES. The naming of chitin nanocrystals was added in section of 'ultrasonication treatment' instead.

Line 96: in which solvent the treated chitin was suspended prior to ultrasonication?

Response: The chitin was pretreated by different DESs and washed with DI water to neutral conditions. All the samples were diluted with DI water prior ultrasonication.

Line 106: authors report they have measured suspension transmittance from 200 to 800 nm. However, it is not clear which wavelenght is used to quantitatively compare the transmittance. Is it 800 nm only?

Response: The chitin suspensions were scanned range from 200 to 800 nm and the result was shown in Figure 1a. The transmittance of ChNCs suspensions in the range from 400 to 800 nm could be used compare the transmittance quantitatively.

Lines 111-112 require revision.

Response: It was rewritten as ' A droplet of ChNCs suspension was spread onto the surface of the newly cut mica and dried under room temperature.'

Results and discussion

Figure 1. I suggest the authors to replace Figure 1 with a Table, where, for every nanocrystal produced, should be reported yield, transmittance at 800 nm (if this is the important one, as the authors seems to imply), and degree of substitutions, as calculated by authors. I suggest to place the picture of the suspensions in the Supplementary Materials. The authors, in Figure 1b, indicate an error bar for each yield. Were the extractions performed in replicate?

Response: We prefer to show the UV-vis spectrophotometer results with the appearance of chitin nanocrystals suspensions as they clearly indicate the quality of suspensions. The degree of substitution could be calculated by FT-IR result, but as only lactic acid is monocarboxylic acid, this method is only applicable for the lactate chitin. For di- or tri-carboxylic acid, this is an inaccurate method as discussed in our manuscript.

The extractions were done in triplicate.

Line 140: "the opaque suspension of ... may be ascribed to inefficient esterification, since their accessibility varies". What is this "their" the authors refer to?
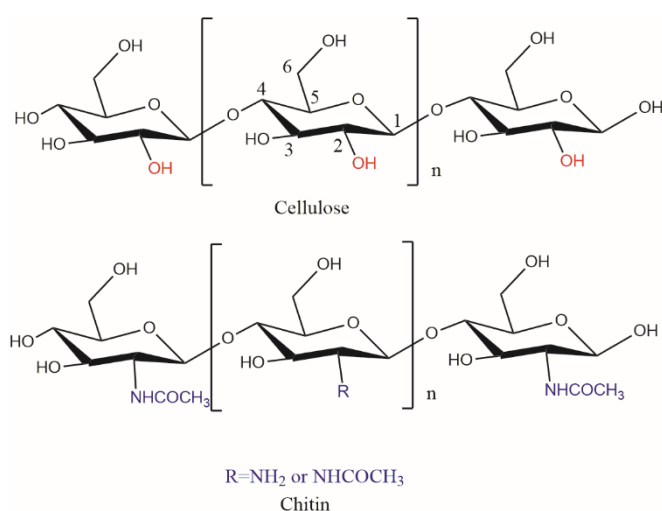
Response: Their refer to acidic DESs. We have rewritten this sentence and made it clearer.

Lines 146-147 are not clear.

Response: We have revised it as 'Thus, to achieve a stable chitin nanocrystals suspension is a premise especially for making composites in water.'

Line 154: in which sense the structure of chitin resembles that of cellulose?

Response: They are both natural polysaccharides having many similarities in the chemical structure. The structure of cellulose and chitin is shown below. The hydroxyl group on C6 in cellulose and chitin can be reacted in similar reactions.

Cellulose

R=NH₂ or NHCOCH₃
Chitin

Which transmittance is considered the minimum acceptable to consider the nanocrystals properly suspended?

Response: It is difficult to define a certain limit for the nanocrystal suspensions. However, comparison of transmittance of different suspensions measured in same conditions clearly indicate

In Section 3.2, the term "peak" should be replaced by "band" or "vibration".
Response: We have revised this part.

Line 175. 2500-400 cm-1 is not fingerprint area
Response: We have rewritten this part.

Line 184. As correctly mentioned in line 181, the band at 1734 cm^{-1} is an overlap of carboxyl and ester.
Response: We have revised this part.

Line 185. Could authors tell where in Zou & Khor, 2005 is stated that the DS of chitin can be obtained by the ratio of carboxyl and amide bands? Which baseline was substracted to obtain band intensity?
Response: We have corrected this reference. It should be 'Feng M et al. Direct conversion of shrimp shells to O-acylated chitin with antibacterial and anti-tumor effects by natural deep eutectic solvents[J]. Green chemistry, 2019, 21(1): 87-98.'.

Line 198 should be revised in style.
Response: We have revised this part.

Figure 2. I suggest to report the spectra as absorbance instead of transmittance. The IR region of interest should be 2000-500 cm^{-1}.
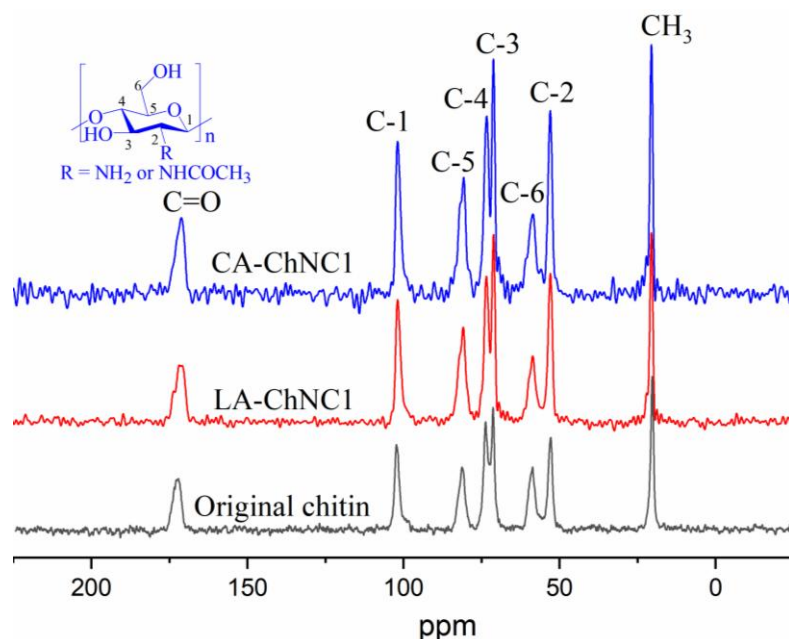Response: We have revised this Figure.

I suggest the authors to calculate the second derivate of the recorded spectra and check whether vibrations for carboxyl, carboxylate anion and ester are cleary visible and separated. The second derivatives could be reported as Supplementary Material.
Response: Since the degree of substitution is very low, the ester and carboxyl group related bands are very weak, and it is hard to conduct that analysis.

Authors should provide more information regarding the formation of ester bonds between chitin and organic acid. 13C-CPMAS spectra of pristine chitin and ChNCs should be provided.
Response: Due to the low degree of esterification of chitin, the related C signal in solid state 13C NMR is hard to detected. We have tried to use 13C-CPMAS to confirm the related groups. The results were shown below (original chitin, LA-ChNC1, and CA-ChNC1 samples).
The chitin samples were characterized by 13C cross-polarization/magic angle spinning (13C CP/MAS). The spectra were acquired with a Bruker AVANCE 400WB spectrometer operating at 100.7 MHz for 13C. The spinning rate was 7.0 kHz, with a recycle delay of 5 s. The sample was acquired using a cptppm2 pulse program using 90° pulses with a strength of 4.0 μs for matched spin-lock cross-polarization transfer with a contact time of 1000 μs and 1000 scans.

Line 218. What do authors mean by stating that oxalic acid- and malic acid-based DES treatment didn't cause significant hydrolysis?

Response: We removed this statement.

Lines 225-231. Why there is a discussion on dispersibility of ChNCs in water in the Morphology section?

Response: We removed this discussion from morphology section and added it into other related section.

Is the aspect ratio related to surface functionalization (line 228)? The discussion on the crystal dimensions could be expanded, it is not clear whether the results obtained are meaningful for application. In Table 2, are the differences in lenght and diameter significant?

Response: The aspect ratio is not related with the surface functionalization. We expanded the discussion on dimension of chitin nanocrystals. The length to diameter ratio is important for ChNCs' application, especially when used as reinforcement in polymer composites. The higher aspect ratio, the better mechanical properties composites can obtain.

Lines 249-252. Does a difference in crystal lenght explain the difference in CrI that has been noticed between acylated and O-acetylated (previous studies) chitin? Because in lines 255-256 the authors correlate the differences in CrI among the obtained ChNCs to their differencies in crystal lenght.

Response: The CrI value is related to the amorphous area and the interactions between the molecule chains. Hydrolysis of chitin in acidic DES can decrease the amorphous area, which can lead to the increase of CrI value of chitin. On the other hand, the O-acylation of chitin may decrease the interaction of chitin molecule chains and decrease the CrI value. Thus, both of these effects may affect CrI value of chitin nanocrystals. Due to the hydrolysis of chitin in OA-ChNC3 and DA-ChNC3, the obtained ChNCs showed a shorter length than others. Thus, a high

amorphous area in chitin indicates a lower CrI value.

Line 273: authors should replace "degradation" with "weight loss (%)", to make clear the discussion is focusing on TGA curves. Are the differences in weight loss reported at line 272 significant?

Response: We replaced 'degradation' with 'weight loss (%)'. There is no significant difference between pristine chitin and ChNCs. The weight loss of ChNCs was slight lower than pristine chitin during this stage, especially for MA-ChNC1, CA-ChNC1.

Line 281: authors could expand the discussion by taking into account DS or other properties of the ChNCs

Response: Many factors can affect the thermal stability of chitin, for example, molecule weight, degree of crystallinity, particle size and introduced functionalized groups on chitin. Because we didn't conduct any more detailed experiments with the samples, it is hard to further discuss accurately the differences of the thermal stability of these five ChNCs.

Line 282: there is no clear connection between the discussion of the DTGA and the application of NCs as reinforcements for melt compounding. Authors should review lines 282-287.

Response: In melt compounding, natural nanoparticles such as chitin and cellulose nanocrystals, can be used as environmentally friendly reinforcements. The processing temperature for thermoplastics can be very high as discussed in the manuscript. Therefore, the thermal stability of ChNCs is important. If ChNCs has poor thermal properties, it may be degraded during mixing or co-extrusion with thermoplastic polymers.

The insert of Figure 5b (zoom on maximum decomposition rate) is not informative as such. In Figure 5b, DTG axis measurement unit should be %/ºC

Response: We have revised this Figure.

Conclusion
Revise English language of lines 294 and 307.

Response: We have rewritten the conclusion part. Moreover, the language was proof-read using Scribendi.

Reviewer #4:

1. Line 38 - Provide the full name of TEMPO first before using abbreviation.
Response: We have added the full name of TEMPO.

2. Why authors did not carry out the surface area and pore size analyses?
Response: BET method was used to analyze the pore size distribution, surface area and size of porous materials. We provided only size distributions, i.e. length and diameter of ChNC since the porosity of the nanoparticles is typically very low. The surface area of non-porous materials is in turn directly related to size distribution.

3. Figure 1 - It might not be interesting to look at the photo of chitin suspension in Figure 1(a). Perhaps the photograph is split and write as (b)
Response: The photos of chitin suspensions in Figure 1(a) were used to show the transmittance and quality differences between the ChNCs suspensions.

4. Line 174 - It is more accurate to say FTIR as a technique to predict the functional groups in a compound, not the chemical structure. In the methodology section, the authors stated the FTIR sample was scanned from 400 to 4000 cm^{-1}. But Figure 2 only displayed the wavenumber up to 2500 cm^{-1}.
Response: We have changed the statement as 'FTIR analysis was conducted to predict the functional groups and the change of the chemical structure of chitin nanocrystals.'

We insert the full spectra in the revised manuscript.

5. Lines 177-179. Too many citations here, please reduce.
Response: We deleted some citations.

6. Line 289 - the title of this caption should have (a) for TGA and (b) for DTG
Response: We have revised it.

7. Line 325 - provide the page range for this article
Response: We added pages in this reference.

8. Line 388 - the name Vespa crabro should be typed using italics.
Response: We have revised it.

9. Line 413 - Is this a book or book chapter?
Response: It is a book.

10. Line 431 - replace the DOI number with volume and page number.
Response: We have revised it.

Reviewer #5:

This manuscript described the production of chitin nanocrystals using acidic deep eutectic solvents. The authors claimed that these nanocrystals were thermally and structurally more stable compared to that of pristine chitin. Also, the acidic DES-systems proved to be simple, effective, and greener approaches to prepare chitin nanocrystals in a one-step reaction. In general, the studies seem to have been well performed, and the article is well prepared. However, the following comment has to be addressed by the authors.

Comments:

1. Figure. 1b, axis label, and the percentage of yield numbers look too blurry to read in the reviewers' copy of the manuscript, probably due to the small lettering of the axes. A bigger version would be preferable, if possible.

Response: A bigger version was added.

2. The authors should prepare a scheme with the molecular representations that justify these interactions between hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) groups with the help of FTIR results.

Response: FTIR is typically used to justify the interactions between HBA and HBD of DES. Actually, this was already studied and reviewed by previous works (Francisco et al. 2012, Francisco et al. 2013). Since we used here DESs just solvents to prepare nanomaterials, we believe that the reader having an interest for the interactions can refer the original publications.

Francisco M, van den Bruinhorst A, Kroon M C. New natural and renewable low transition temperature mixtures (LTTMs): screening as solvents for lignocellulosic biomass processing[J]. Green Chemistry, 2012, 14(8): 2153-2157.

Francisco M, van den Bruinhorst A, Kroon M C. Low- transition- temperature mixtures (LTTMs): A new generation of designer solvents[J]. Angewandte Chemie international edition, 2013, 52(11): 3074-3085.

3. Line 229 and 230, the author stated that the malate chitin reported for antibacterial and better antitumor activity compared to pristine chitin. It would be better to provide experimental data of prepared ChNC for the same.

Response: This statement was related to previous study (Feng, et al. 2019). We didn't make similar experiments by ourselves.

Feng M, Lu X, Zhang J, et al. Direct conversion of shrimp shells to O-acylated chitin with antibacterial and anti-tumor effects by natural deep eutectic solvents[J]. Green chemistry, 2019, 21(1): 87-98.

4. In Figure 5, the axis of the graph is very small and not readable (expanded view of DTG curves) and provides a higher resolution image.

Response: We have added a clearer one in the revised version.

5. It would be interesting if the authors can comment on the eco-benign advantages of DES system in contrast with the hydrochloric acid hydrolysis method with experimental data.

Response: For the traditional HCl hydrolysis method, the yield has been reported to range from 40%

to 86% (Araki et al. 2012, Ma et al. 2014, Phongying et al. 2007). The typical concentration of HCl has been 3 M, and the reaction temperature and reaction time have varied between 80 °C and 104 °C and 1.5–6 h, respectively (Salaberria et al. 2015). In current study, the reaction time was only 1 h at 100 °C for choline chloride and lactic acid DES, and the yield was up to 87.5%. Moreover, choline chloride and lactic acid are natural products, which are also sustainable and nontoxic. Therefore, we consider that the DESs have many eco-benign advantages compared with HCl method in the production of ChNCs.

Araki J, Yamanaka Y, Ohkawa K. Chitin-chitosan nanocomposite gels: reinforcement of chitosan hydrogels with rod-like chitin nanowhiskers[J]. Polymer journal, 2012, 44(7): 713.

Ma B, Qin A, Li X, et al. Structure and properties of chitin whisker reinforced chitosan membranes[J]. International journal of biological macromolecules, 2014, 64: 341-346.

Phongying S, Aiba S, Chirachanchai S. Direct chitosan nanoscaffold formation via chitin whiskers[J]. Polymer, 2007, 48(1): 393-400.

Salaberria A M, Labidi J, Fernandes S C M. Different routes to turn chitin into stunning nano-objects[J]. European Polymer Journal, 2015, 68: 503-515.

6. The manuscript needs to be CAREFULLY proofread

Response: The manuscript was proof-read using a commercial proof-reading service (Scribendi).

Reviewer #6:

The paper entitled 'Comparison of acidic deep eutectic solvents in production of chitin nanocrystals' is an interesting study towards hydrolysis of chitin for applications. However, there are few observations which demand to be included or justification required in the manuscript for publication in the journal 'Carbohydrate Polymers':

(i)   In the high lights please mention what functional groups were introduced, not a general statement, keeping in mind the number of words.

Response: We revised it.

(ii)   Line 29 and 30; individualization of nanoscale entitities…:   Please justify individualization at this place. Further in solutions—which solution is used?

Response: "Individualization" refers to dispersing chitin into individual nanofibers or nanocrystal with a size on the nanometer level. We have specified the solution as water.

(iii)   Line 43: In addition of the groups: which groups ? please make a correct sentence

Response: We revised it.

(iv) Line 48, 49 reconstitute the sentence (a notably, is formed?)

Response: We have reconstituted this sentence.

(iv)   What is the difference of nanocrystals of chitin in isolation of ChNc using DES, choline chloride and thiourea and acids used in the present investigation? Please justify.

Response: Chitin nanofibers (ChNFs) prepared in choline chloride and thiourea had width of 25–45 nm and length of 162–450 nm. ChNCs prepared in choline chloride and acids had higher width (OA-ChNC3 of width 49±8 nm, LA-ChNC1 of width 44±11, MA-ChNC1 of width 47±10, CA-ChNC1 of width 42±10 and DA-ChNC3 of width 49±10); and some ChNCs had lower length (LA-ChNC1 of length 367±48, MA-ChNC1 of length 257±59, CA-ChNC1 of length 306±58), and some ChNCs had higher length (OA-ChNC3 of length 530±89, and DA-ChNC3 of length 670±92). The ChNCs prepared in this paper has better size uniformity. We also tried to use choline chloride and thiourea DES for dissolving and regenerating chitin. However, after ultrasonication treatment, the regenerated chitin couldn't form well dispersed suspension. This may be due to the chitin source or the sonication instrument. The ChNC from choline chloride and thiourea showed a milky appearance and the micromorphology showed a serious aggregation of individual ChNCs. Therefore, acidic DESs are more efficient than previously reported DESs in the production of ChNCs.

(v)   In the whole manuscript DES, DESs and ChNc and ChNCs are written, please write correct world wherever required. For e.g. Line 58, 65, 68 etc.

Response: We revised it.

(vi)   Section 2.4 and 2.5 not clear for e.g. Line 96 never-dried chitin? It is pretreated chitin? Please justify. …. To a concentration of 0.5%?   Please mention the solvent.

Response: We revised it as "The pretreated never-dried chitin sample was diluted to a

concentration of 0.5 wt% with deionized water…".

(vii)   Section 3.1 Line 146. Please mention applications with references
Response: We have added related reference.

(viii)   Fig 1a is not clear, whether it has any significance in the manuscript?
Response: Fig 1 directly shows whether the ChNCs can form a stable dispersion in water suspension together with the UV curves.

The transparency of chitin nanocrystals is important for its potential applications in transparent packaging coatings, films and laminates (Zhong et al. 2019).
Zhong, T., Wolcott, M. P., Liu, H., & Wang, J. (2019). Developing chitin nanocrystals for flexible packaging coatings. CARBOHYDRATE POLYMERS, 226, 115276.

(ix)   Line 158: over hydrolysis of amorphous region Please define the statement.
Response: Over hydrolysis of amorphous region means that too much amorphous region in chitin was hydrolyzed.

(x)   In the manuscript, maleic acid and malic acid are used please maintain uniformity throughout the text.
Response: Maleic acid and malic acid are two different acids, the type of malic acid used in this article is DL-malic acid.

(xi)   DS was calculated only for two samples; however the products are prepared more than two. Further, whether FTIR was carried out quantitatively to measure the absorption ratio of the peaks?
Response: FTIR could be used for calculating the DS according to related references (Mine et al. 2009, Feng et al. 2019). The absorption at 1734 cm^{-1} is related to ester and carboxyl groups, thus this method is applicable only for monocarboxylic acid, i.e. lactic acid. The others were described as absorption ratio.
Mine S, Izawa H, Kaneko Y, et al. Acetylation of α-chitin in ionic liquids[J]. Carbohydrate research, 2009, 344(16): 2263-2265.
Feng M et al. Direct conversion of shrimp shells to O-acylated chitin with antibacterial and anti-tumor effects by natural deep eutectic solvents[J]. Green chemistry, 2019, 21(1): 87-98

(xii)   Line 194 to 202: Obvious flocculation --; inefficient esterification; and it was easier…; extra carboxylic acid groups. Please justify these statements/ correct the sentences.
Response: Obvious flocculation means some larger particles formed in chitin suspension during storage, which means the instability of ChNCs suspension.
Inefficient esterification means the degree of substitution is relative lower in OA-ChNC1 and DA-ChNC1.
'it was easier' was changed to '…it was **easy** form ester group with amorphous area of chitin **first** and…'.
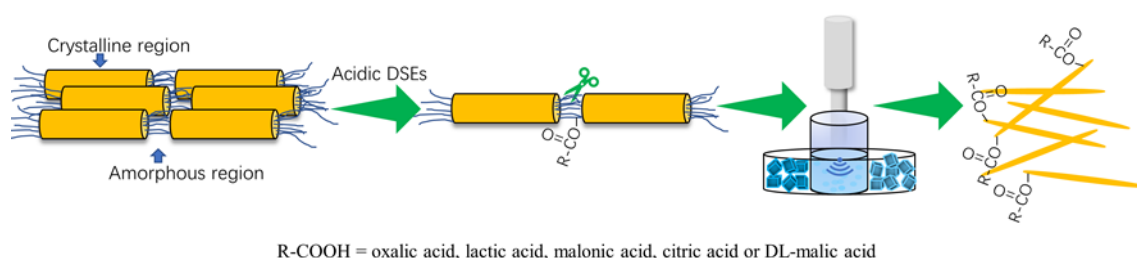We deleted 'extra'.

(xiii) Further, why lactic acid is more accessible to amorphous area of chitin in comparison to other acids. Please justify.

Response: During the hydrolysis reaction of chitin the amorphous regions were dissolved firstly and then the crystalline regions. Previous study (Qi et al. 2013) found that lactic acid is more active to the protonate chitin. Therefore, we deduced that lactic acid is more accessible to amorphous area of chitin in comparison to other acids.

Qi Z D, Fan Y, Saito T, et al. Improvement of nanofibrillation efficiency of α-chitin in water by selecting acid used for surface cationisation[J]. RSC Advances, 2013, 3(8): 2613-2619.

(xiv) In the results and discussion part, reaction mechanism of DES and chitin may be given to understand the process in a more scientific way.

Response: Mechanistic illustration for producing ChNCs in organic acid DESs was added to help reader to better understand the process.

R-COOH = oxalic acid, lactic acid, malonic acid, citric acid or DL-malic acid

(xv) Section 3.3: range is specified for nanocrystals and it is mentioned 'diameter of nanocrystals was similar…' please justify the statement.

Response: There is no significant difference in the diameter range of different ChNCs samples prepared by different types of DES used in this paper. We revised this sentence.

(xvi) Line 217 and 218: Please, correct the sentences.

Response: We have corrected the sentences.

(xvii) Line 219: 'malonic acid penetrates in the amorphous region', while in FTIR it is mentioned lactic acid penetrates in the amorphous region. Please justify this.

Response: Malonic acid penetrates also easily in the amorphous region of chitin. The pKa value of lactic is 3.86, higher than that of malonic acid (pKa1 = 2.83). The strong acid may lead to significant hydrolysis of chitin and result in shorter ChNCs.

(xviii) Line 230: (C6 glioma): What is C6 glioma?

Response: It is Rat C6 glioma cells. We revised this.

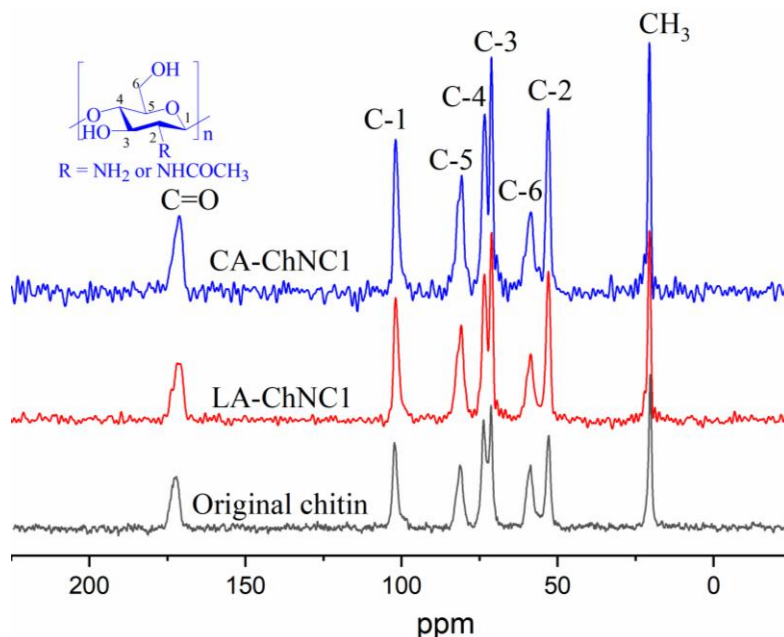(xix) Applications of prepared nanocrystals may be included in the conclusion part.

Response: We included the possible application of prepared nanocrystals in conclusion part.

(xx) Further, the prepared nanocrystals may also be characterized by NMR spectroscopy techniques?

Response: Due to the low degree of esterification of chitin, the related C signal in solid state 13C

NMR is hard to detected. We have tried to use 13C-CPMAS to confirm the related groups. The results were shown below (original chitin, LA-ChNC1, and CA-ChNC1 samples).

The chitin samples were characterized by 13C cross-polarization/magic angle spinning (13C CP/MAS). The spectra were acquired with a Bruker AVANCE 400WB spectrometer operating at 100.7 MHz for 13C. The spinning rate was 7.0 kHz, with a recycle delay of 5 s. The sample was acquired using a cptppm2 pulse program using 90° pulses with a strength of 4.0 µs for matched spin-lock cross-polarization transfer with a contact time of 1000 µs and 1000 scans.

(xxii) The nanocrystals prepared from acidic hydrolysis as given in literature and with eutectic solvents, what is the basic difference between the two methods in relation to structure and their application part. Please justify.

Response: The morphologies of ChNC from the HCl and DESs methods didn't show too much difference. However, the ChNCs prepared with HCl is not stable in neutral water suspension, due to the low surface charge. It can form stable suspension in acidic condition (as shown below). This may limit its use in many applications. The ChNCs in turn showed a highly stable suspension in neutral condition (Figure 1a). They may be suitable as such in many applications such as emulsifiers for Pickering emulsions.
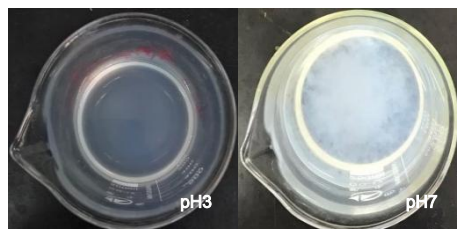

Figure. Suspension of chitin nanocrystal prepared with HCl hydrolysis (Hong, et al. 2019).

Hong S, Yuan Y, Yang Q, et al. Choline chloride-zinc chloride deep eutectic solvent mediated preparation of partial O-acetylation of chitin nanocrystal in one step reaction[J]. Carbohydrate polymers, 2019, 220: 211-218.

Reviewer #8:

1. Highlight no. 2. It will be more meaningful if you add the kind of your production method.
Response: We have added the method in highlight.

2. In introduction, it will be better to give general description about nanocrystals or nanofiber, therefore the reader may imagine the form or characteristics of them.
Response: We added the description of chitin nanocrystals and nanofibers in introduction section.

3. Line 83-86. Is there any reference that you used to synthesize the DESs?
Response: Choline chloride and organic acids interact through hydrogen bond acceptors and hydrogen bond donors, and this mechanism of action has been described in many literatures.
Francisco M, van den Bruinhorst A, Kroon M C. New natural and renewable low transition temperature mixtures (LTTMs): screening as solvents for lignocellulosic biomass processing[J]. Green Chemistry, 2012, 14(8): 2153-2157.
Francisco M, van den Bruinhorst A, Kroon M C. Low- transition- temperature mixtures (LTTMs): A new generation of designer solvents[J]. Angewandte Chemie international edition, 2013, 52(11): 3074-3085.
Zhu, P., Gu, Z., Hong, S., & Lian, H. (2017). One-pot production of chitin with high purity from lobster shells using choline chloride–malonic acid deep eutectic solvent. Carbohydrate polymers, 177, 217-223.
Saravana, P. S., Ho, T. C., Chae, S. J., Cho, Y. J., Park, J. S., Lee, H. J., & Chun, B. S. (2018). Deep eutectic solvent-based extraction and fabrication of chitin films from crustacean waste. Carbohydrate polymers, 195, 622-630.
Shu, H., Yang, Y., Qiuru, Y., Ping, Z., & Lian, H. (2018). Versatile acid base sustainable solvent for fast extraction of various molecular weight chitin from lobster shell. Carbohydrate polymers, 201, 211-217.

4. Line 95. after ultrasonication treatment, is the chitin can be called as chitin nanocrystals? give the statement to clarify how to produce chitin nanocrystals. And in what form you stored the chitin?in wet form or dried form?
Response: After pretreated by DESs, the amorphous region in chitin was hydrolyzed by acidic DESs. The reaction solution was washed to a neutral pH and the samples were collected. Thereafter, ultrasonication treatment was used to liberate the chitin nanocrystals. Thus, the chitin after ultrasonication can be called chitin nanocrystals. The AFM analysis also supported this statement. The samples were collected and stored at 4 °C in a fridge (We mentioned this in Ultrasonication treatment section).

5. In the line 132, please clarify the reaction that you mean. Is this enough with the mixing or any other way to react?
Response: We rewrote this sentence. The reaction condition is mentioned in section of 'Pretreatment of chitin in DESs'. The reaction is heterogeneous, it is enough with mixing.

6. Line 132 chitosan can be dispersed in water. What pH?
Response: This article does not discuss chitosan. It's chitin. The obtained chitin nanocrystals were dispersed in neutral DI water.

7. In method, you may mention what pristine chitin you use? (specific source or the factory that produced it).

Response: We added related information of chitin in section of 'Materials'

8. Figure 3. What does the mean of aspect ratio?

Response: Aspect ratio means the ratio of ChNCs' length to diameter.

Sincerely yours,

Hailan Lian

1   Comparison of acidic deep eutectic solvents in production of chitin

2   nanocrystals

3   *Yang Yuan [a], Shu Hong [a, b], Hailan Lian [a, *], Kaitao Zhang [b], Henrikki Liimatainen [b]*

4   [a] College of Materials Science and Engineering, Nanjing Forestry University, Nanjing,

5   210037 China.

6   [b] Fibre and Particle Engineering Research Unit, University of Oulu, P.O. Box 4300,

7   FI-90014, Finland.

8   **Abstract:** Five different acidic deep eutectic solvents (DESs) composed of choline chloride

9   and organic acids were applied to fabricate chitin nanocrystals (ChNCs). All DESs resulted in

10   high transmittance and stable ChNCs suspensions with very high mass yield ranging from 78%

11   to 87.5% under proper reaction conditions. The acidic DESs had a dual role in ChNCs

12   fabrication, i.e. they promoted hydrolysis of chitin and acted as an acylation reagent.

13   Physicochemical characterization of chitin revealed that the removal of amorphous area during

14   DES treatments led to increased crystallinity of ChNCs and a dimension diversity correlated

15   the DES used. The average diameter and length of individual ChNCs ranged from 42 nm to 49

16   nm and from 257 nm to 670 nm, respectively. The thermal stability of ChNCs was comparable

17   to that of pristine chitin. Thus, acidic DESs showed to be

18   non-toxic and environmentally benign solvents for production of functionalized chitin

19   nanocrystals.

20   **Keywords:** Chitin; Nanocrystals; Deep Eutectic Solvents; Organic acid

[*] *Corresponding author: Hailan Lian (lianhailan@njfu.edu.cn), Tel./fax.: (86-25) 85427531.*

1. Introduction

Chitin is a linear polysaccharide consisting of N-acetyl-D-glucosamine units which are connected by β-1,4-glycosidic linkages. It is the second most abundant biopolymer after cellulose on the earth, and exists in insect exoskeletons (Zhang, Haga, Sekiguchi & Hirano, 2000), crustacean shells (Hong, Yang, Yuan, Chen, Song & Lian, 2019a), cell walls of various fungi (Teng, Khor, Tan, Lim & Tan, 2001) and a few algae (Roberts, 1992; Salah et al., 2013). Owing to its biocompatibility, biodegradability and non-toxicity, chitin is used in different fields such as in food (Shahidi, Arachchi & Jeon, 1999), biomedicine (Ehrlich et al., 2010), agriculture (Ramírez, Rodríguez, Alfonso & Peniche, 2010), tissue engineering (Freier, Montenegro, Koh & Shoichet, 2005) and environmental applications (Aranaz et al., 2009). Only a few solvents, for example, NaOH/urea, ionic liquid and N-N-Dimethylacetamide/LiCl can be used for dissolving chitin for further applications (Barber, Griggs, Bonner & Rogers, 2013; Hu et al., 2007; Terbojevich, Carraro, Cosani & Marsano, 1988; Xu, Zhang, Duan & Chen, 2017). However, the insolubility of chitin in water and most organic solvents limits its use in many end-uses. Therefore, individualization of nanoscale entities from chitin can enhance homogeneous dispersion of chitin in water and expand its feasibility in novel applications (Muzzarelli, Mehtedi & Mattioli-Belmonte, 2014).

Nanochitin, i.e. chitin nanocrystals (ChNCs, having diameter less than 100 nm and length less than 1 μm) and chitin nanofibers (ChNFs, having diameter less than 100 nm and length over 1 μm) (Liu, Zhu, Li, Tian, Chen & Chen, 2013b), has a high aspect ratio and superficial area, low density, good mechanical properties and is biocompatible. It has been used e.g. in polymer reinforcement (Araki, Yamanaka & Ohkawa, 2012), tissue engineering (Li, Li, Wang, Gu, Jiang & Wu, 2014), drug delivery (Azuma, Ifuku, Osaki, Okamoto & Minami, 2014), hemostasis and

44   wound healing (Naseri, Algan, Jacobs, John, Oksman & Mathew, 2014). ChNCs are typically

45   produced by mineral acid hydrolysis (e.g. HCl) of chitin at high temperature, but also other

46   methods can be used (Araki et al., 2012; Ma, Qin, Li, Zhao & He, 2014; Phongying, Aiba &

47   Chirachanchai, 2007). For instance, TEMPO (2, 2, 6, 6-tetramethylpiperidin-1-yloxyl) mediated

48   oxidation reacts and solubilizes the amorphous parts of chitin and results in ChNCs with

49   sonication treatment, while surfaces of crystalline section (C6 hydroxyl) are oxidized to

50   carboxylic acid or carboxylate (Fan, Saito & Isogai, 2008). Similar, alkaline conditions can also

51   promote dissolution of amorphous parts and partial deacetylation of chitin (Fan et al., 2008; Fan,

52   Saito & Isogai, 2009, 2010). Subsequently, the partially deacetylated chitin was dispersed in acidic

53   condition, the protonation of NH_2 in C2 of chitin in acidic conditions creates internal electrostatic

54   repulsion within chitin chains and can enhance liberation of ChNCs. Besides, esterification of the

55   hydroxyl groups at C3 and C6 with maleic anhydride can also be used to produce carboxylated

56   ChNCs (Aklog, Nagae, Izawa, Morimoto, Saimoto & Ifuku, 2016; Wang, Yan, Chang, Ren &

57   Zhou, 2018).

58   Deep eutectic solvents (DESs) are a class of green solvents which are typically formed by

59   mixing a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) under a certain

60   temperature. The formed clear solution has a notably lower melting than that of its individual

61   constituents. DESs are often considered as a sub-class of ionic liquid, because of their many

62   similar physical and chemical properties such as low vapor pressure and good solvent capacity

63   (Zhang, Vigier, Royer & Jerome, 2012). In addition, DESs have many superior properties such as

64   simple synthesis route, non-toxicity, recyclability and biodegradability. Previously DESs derived

65   from quaternary ammonium salts and carboxylic acids have been used for processing of different

66   biopolymers (Abbott, Boothby, Capper, Davies & Rasheed, 2004). For example, DESs based on

67   choline chloride and carboxylic acids were used for extraction of cellulose (Liu et al., 2017a),

68   production of nanocellulose (Liu et al., 2017a; Liu et al., 2017b; Sirviö, Visanko & Liimatainen,

69   2016) and extraction of chitin (Hong, Yuan, Yang, Zhu & Lian, 2018; Zhu, Gu, Hong & Lian,

70   2017). Moreover, DES based on choline chloride and thiourea have been harnessed in isolating of

71   chitin nanofibers (Mukesh, Mondal, Sharma & Prasad, 2014) and chitin nanocrystals (Cao et al.,

72   2019; Hong et al., 2019b). However, DESs composing of choline chloride and organic acids,

73   which are known to be efficient in hydrolysis of cellulose and preparation of cellulose

74   nanocrystals (Sirviö et al., 2016), have not reportedly used in ChNCs isolation. Therefore, it is not

75   astonishing that choline chloride and organic acids may be used as greener hydrolytic medium for

76   production of ChNCs. But due to the difference of molecular structure between cellulose and

77   chitin, the results may differ. Thus, it is essential to study organic acids based DESs as promising

78   media for preparation of ChNCs.

79   Here DESs derived from choline chloride and five different organic acids (oxalic acid, lactic

80   acid, malonic acid, citric acid and DL-malic acid) were compared in fabrication of ChNCs in an

81   ultrasound assisted process. The morphology of the synthesized ChNCs was observed through

82   atomic force microscope (AFM). The physicochemical characteristics of ChNCs were studied by

83   Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and thermogravimetric

84   analysis (TGA).

85   **2. Materials and methods**

86   *2.1 Materials*

87   Oxalic acid dihydrate and citric acid monohydrate were purchased from Nanjing Chemical

88   Reagent Co., Ltd. (Nanjing, China). Choline chloride and malonic acid were obtained from

89   Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), lactic acid from Shanghai Lingfeng

90   Chemical Reagent Co., Ltd and DL-malic acid from Shanghai Macklin Biochemical Co., Ltd.

91   (Shanghai, China). Chitin from crab shell was purchased from Shanghai Aladdin Biochemical

92   Technology Co., Ltd. (Shanghai, China). Deionized water was used throughout the whole

93   experiment. All chemicals were used without any further purification.

94   *2.2 Synthesis of DESs*

95   Choline chloride was mixed with organic acid (oxalic acid dihydrate, lactic acid, malonic

96   acid, citric acid monohydrate or DL-malic acid) in the conical flask with the molar ratio of 1:2 at

97   100℃ in oil bath with continuous magnetic stirring until the mixture become transparent and

98   homogeneous. The used DES systems are summarized in Table 1.

99   **Table 1**
100   DESs used for production of chitin nanocrystals. Molar ratio between HBA and HBD was 1:2 in
101   all DESs.

DES	HBA	HBD
CCOA	Choline chloride	Oxalic acid dihydrate
CCLA	Choline chloride	Lactic acid
CCMA	Choline chloride	Malonic acid
CCCA	Choline chloride	Citric acid monohydrate
CCDA	Choline chloride	DL-malic acid

102   *2.3 Pretreatment of chitin with DESs*

103   Chitin powder (2g) was treated in DESs (50 g) under mixing at 100℃ for 1h and 3h,

104   respectively. The reaction was quenched by adding deionized water and the treated solids was

105   washed with water using centrifugation at 4000 rpm for 5 min until the suspension became

106   neutral.

5

2.4 Ultrasonication treatment

The pretreated never-dried chitin sample was diluted to a concentration of 0.5 wt% with deionized water, and the suspension was sonicated with Ultrasonic Cell Crusher ATPIO-1200D (Nanjing Xianou Instruments Manufacture Co. Ltd. Nanjing, China) at output power of 1000 W and 2/2 s on/off pulses for 30 min. The samples were collected and stored at 4 °C in a fridge for further analysis and denoted as OA-ChNC1, OA-ChNC3 (pretreated with CCOA for 1h and 3h); LA-ChNC1, LA-ChNC3 (CCLA 1h and 3h); MA-ChNC1, MA-ChNC3 (CCMA 1h and 3h); CA-ChNC1, CA-ChNC3 (CCCA 1h and 3h); DA-ChNC1, DA-ChNC3 (CCDA 1h and 3h), respectively.

2.5 Ultraviolet-visible spectrophotometry

Transmittance of DES-treated chitin suspensions was measured by a UV-vis spectrophotometer (TU-1080, Beijing Purkinje General Instrument Co., Ltd, China) in the range from 200 nm to 800 nm. The samples were diluted to a concentration of 0.3 wt-%, and then sonicated at 500 W for 2 min with a cell disrupting ultrasonic analyzer. Pure water was used as a control.

2.6 Atomic force microscopy (AFM)

The samples were diluted into a 0.001 wt-% aqueous solution and sonicated for 1 minute. A droplet of ChNCs suspension was spread onto the surface of the newly cut mica and dried under room temperature. After that, the images (5×5 μm^2) were acquired in tapping mode with silicon nitride cantilever tips by AFM (Bruker Dimension Icon AFM, Bruker, Billerica, MA). Images were processed and analyzed with the NanoScope Analysis 1.8 software.

2.7 Fourier transform infrared spectroscopy (FTIR)

129   VERTEX 80V FT-IR spectrometer (Bruker, Germany) with a range of 4000-400 cm^{-1} (4 cm

130     $^{-1}$ resolution) was used to acquire the FT-IR spectra samples. KBr technique (2 mg sample per 100

131   mg KBr) was applied.

132   *2.8 X-ray powder diffractometry (XRD)*

133   The XRD analysis was conducted on a Bruker D8 ADVANC (Germany) using a copper

134   target λ = 1.5406, a tube voltage of 40 kV, a tube current of 30 mA, a scan angle range of 2θ = 5 to

135   55°, and a scan speed of 5°/min. The crystalline index (CrI; %) was calculated according to Eq. (1)

136     $$CrI_{110} = [\frac{I_{110}-I_{am}}{I_{110}}] \qquad (1)$$

137   where I_{110} is the maximum intensity at $2\theta \cong 20°$ and I_{am} is the intensity of amorphous

138   diffraction at $2\theta \cong 16°$ (Focher, Beltrame, Naggi & Torri, 1990).

139   *2.9 Thermogravimetry analysis (TGA)*

140   The NETZSCH STA 449C (Netzsch, Selb, Germany) analyzer was used to analyze TG and

141   DTG curves with a heating rate of 10℃ min^{-1} in a nitrogen atmosphere at 35 ~ 600℃.

142   **3. Results and Discussion**

143   *3.1 Optical properties and mass yield of ChNCs suspensions*

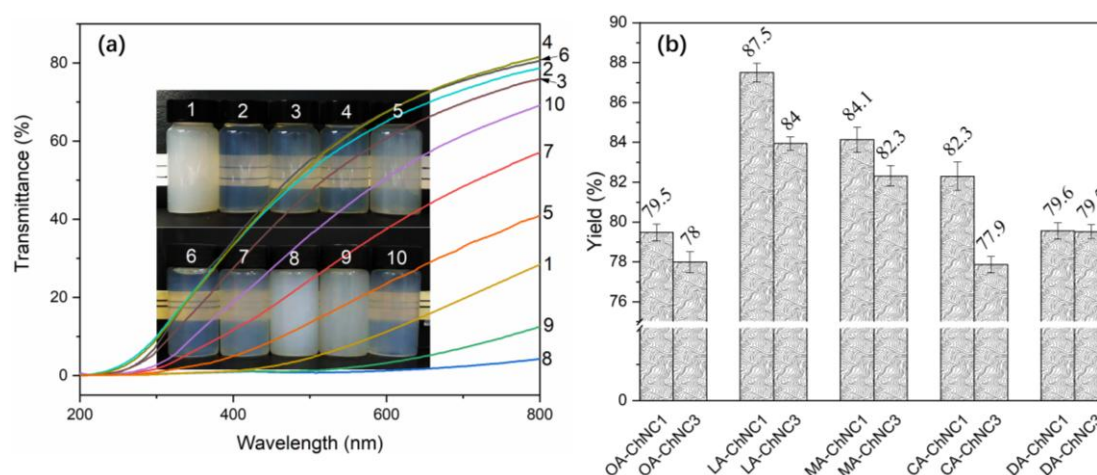144   After ultrasound treatment, the chitin nanocrystals pretreated by all acidic DESs systems at

145   100 ℃ for 1 h were uniformly dispersed in water and formed stable suspensions (**Figure 1a**).

146   However, the transmittance of LA-ChNC1, MA-ChNC1, and CA-ChNC1 (78.6%, 75.9%, 81.5%

147   at 800 nm, respectively) was much higher than that of OA-ChNC1 and DA-ChNC1 (28.4% and

148   40.9% at 800 nm, respectively) indicating the differences in particle size distributions. The

149   carboxylic acids used here as second components of DESs are able to react with hydroxyl groups

150 of chitin and form ester bonds. Previous work has shown that ester groups can in turn serve as a

151 steric barrier that blocks hydrogen bonding between the chitin chains, and enhance chitin

152 disintegration to nanocrystals (Hong et al., 2019b). The opaque suspension of OA-ChNC1 and

153 DA-ChNC1 may be ascribed to inefficient esterification reaction, since different acidic DESs'

154 accessibility towards polysaccharides varies (Hong et al., 2018; Xu, Ding, Han, Dong & Ni, 2016).

155 Therefore, chitin may be only partly disintegrated during ultrasonication treatment. On the other

156 hand, DES based on lactic acid, which is a monocarboxylic acid, resulted in a highly transparent

157 ChNCs suspension. It is likely that hydrophilicity of lactate groups promoted formation of stable

158 aqueous suspension (Araki & Kurihara, 2015). Unlike the chitin nanocrystals from traditional HCl

159 hydrolysis, the obtained ChNCs from the DES treatments exhibited excellent dispersibility in

160 water at neutral water condition. This feature can also be beneficial for many applications of

161 ChNCs in which stable water suspensions are required (Liu, Zhu, Li, Tian, Chen & Chen, 2013).

162 Thus, to achieve a stable chitin nanocrystals suspension is premise especially for making

163 composites in water and for water treatment.

164   To improve the grafting efficiency and colloidal stability of ChNCs suspensions, the DESs

165 treatment time was extended to three hours. Interestingly, the transmittance of both OA-ChNC3,

166 and DA-ChNC3 (80.5%, 69.1% at 800 nm, respectively) increased with the extended reaction

167 time, while MA-ChNC3 and CA-ChNC3 (4.3%, 12.5% at 800 nm) suspensions were milky white

168 and almost opaque (**Figure 1a**). Previously, it has been noted that esterifying agents including

169 carboxylic acids and carboxylic anhydrides can results in cross-linking of cellulose chains (Sirviö

170 et al., 2016). The structure of chitin resembles that of cellulose, and the hydroxyl groups of chitin

171 are capable to react with di- or tri- carboxylic acid (maleic acid and citric acid) and can form

172 aggregated structures in suspensions. It was also found that the transmittance of LA-ChNC3 (57.1%

173 at 800 nm) was lower than that of LA-ChNC1. The possible explanation for this result is likely the

174 over hydrolysis of amorphous region of chitin and low degree of esterification of chitin.

175   As shown in **Figure 1b**, the mass yield of ChNCs varied from 77.9-87.5%, and was the

176 highest with LA-ChNC1 (87.5%). The yield of all ChNCs decreased as the chitin exposed to more

177 severe hydrolysis reaction (1h vs. 3 h reaction time) with all DESs systems, except with that of the

178 DA-ChNCs (**Figure 1b**). Presumably, the hydrolysis reaction was suppressed because of the

179 esterification of the hydroxyl groups of chitin through formation of monoester or diesters (Sirviö

180 et al., 2016). The yield of ChNCs prepared through HCl hydrolysis is typically ranging from 40%

181 to 86% depending on reaction time and source of chitin (Araki et al., 2012; Ma et al., 2014;

182 Phongying et al., 2007). Therefore, the yield in DES treatments was similar or better than in

183 traditional mineral acid hydrolysis.

184

185   **Figure 1.** a) UV–vis spectra and b) yield of ChNCs suspensions after ultrasonication
186 treatment. The inserts are photographs of corresponding chitin suspensions: (1) LA-ChNC1, (3)
187 MA-ChNC1, (4) CA-ChNC1, (2) LA-ChNC1, (3) MA-ChNC1, (4) CA-ChNC1, (5) DA-ChNC1,
188 (6) OA-ChNC3, (7) LA-ChNC3, (8) MA-ChNC3, (9) CA-ChNC3, (10) DA-ChNC3.

189 *3.2 Chemical characteristics of ChNCs*

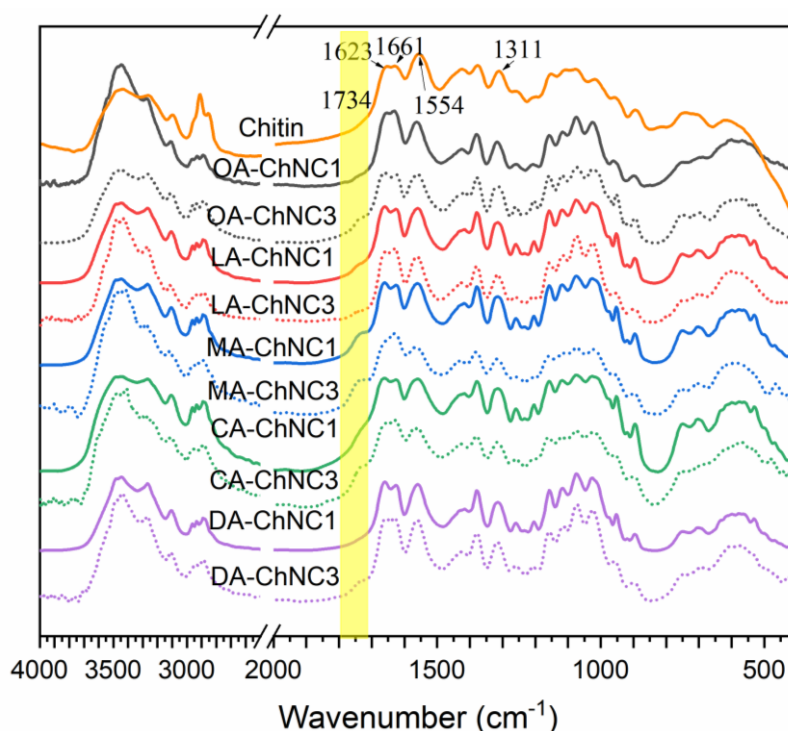190   FTIR analysis was conducted to predict the functional groups and the change of the chemical

191 structure of chitin nanocrystals. The characteristic bands of chitin nanocrystals and pristine chitin

192 in 4000-400 cm^{-1} region, are shown in **Figure 2**. The chitin bands were assigned according to

193 previous reports (Brugnerotto, Lizardi, Goycoolea, Argüelles-Monal, Desbrières & Rinaudo, 2001;

194 Bulut, Sargin, Arslan, Odabasi, Akyuz & Kaya, 2017; Chen, Deng, Yang, Yang, Ye & Li, 2018;

195 Chen, Li, Yano & Abe, 2019; Kaya et al., 2017). Compared with the pristine chitin, all DESs treated

196 chitins showed a new a band around 1734 cm^{-1}, which is attributed to band of the carbonyl

197 vibrations of ester and carboxylic acid group (Hong et al., 2019b; Ifuku, Morooka, Morimoto &

198 Saimoto, 2010). The results revealed that the esterification reaction and hydrolysis reaction

199 proceeded simultaneously. The degree of substitution (DS) of the acylated chitin was assessed by

200 comparing the absorption ratio of ester and carboxyl at 1734 cm^{-1} and the amide I at the 1661 cm^{-1}

201 bands (Feng et al., 2019). Since only lactic acid is monocarboxylic acid, this method is only

202 applicable for lactate chitin. The other organic acids are di- or tri-carboxylic acids, the degree of

203 substitution could be comparatively studied by calculating the absorption ratio of 1734/1661 cm^{-1} to

204 indicate the amount of grafting groups on chitin molecule chains. The DS of the ester groups of

205 LA-ChNC1 and LA-ChNC3 were 0.30 and 0.26, respectively. The absorption ratio of 1734/1661 for

206 OA-ChNC1, OA-ChNC3, MA-ChNC1, MA-ChNC3, CA-ChNC1, CA-ChNC3, and DA-ChNC3

207 were 0.22, 0.41, 0.34, 0.54, 0.34, 0.50, and 0.26, respectively. The band at 1734 cm^{-1} of DA-ChNC1

208 was too weak due to the low substitution to give the accurate value.

209   Another observation was that when the chitin nanocrystals suspensions were stored at room

210 temperature for two weeks, obvious flocculation in OA-ChNC1 and small amount of sediment in

211 DA-ChNC1 was found. This phenomenon confirmed the inefficient esterification of OA-ChNC1

212 and DA-ChNC1. The absorption ratio of 1734/1661 of OA-ChNC3, MA-ChNC3 and CA-ChNC3

213   increased with extended reaction time, and only DS of LA-ChNC3 decreased. A plausible reason

214   for this may be ascribed to fact that lactic acid is easier to cause the protonation of amorphous area

215   in chitin (Qi, Fan, Saito, Fukuzumi, Tsutsumi & Isogai, 2013), and it was easy form ester group

216   with amorphous area of chitin first and extended reaction time may also resulted in over

217   hydrolysis of chitin. This result also gives the explanation of the lower transmittance of

218   LA-ChNC3. The introduced carboxylic acid group could make it be more reactive with other

219   chemicals to fabricate other functional materials (Chen et al., 2017; de Cuadro et al., 2015; He et al.,

220   2018). Moreover, the surface functionalization expands the feasibility of ChNCs from DES

221   treatments in many applications. For example, malate chitin was reported to provide additional

222   antibacterial activity (*Escherichia coli*) and a better antitumor effect (Rat C6 glioma cells)

223   compared with the pristine chitin (Feng et al., 2019).

224   Other characteristic bands located at around 1661 cm^{-1} and 1623 cm^{-1} are assigned to the amide

225   I band, while bands at 1554 cm^{-1} and 1331 cm^{-1} were ascribed to amide II and amide III bands,

226   respectively. The obtained chitin samples were all in the α form after DESs treatment. No other

227   additional bands were observed, indicating that no side reactions had occurred. Taking the energy

228   consumption and the stability of chitin nanocrystals suspensions into account, OA-ChNC3,

229   LA-ChNC1, MA-ChNC1, CA-ChNC1 and DA-ChNC3 were selected for further analysis.

Figure 2. FT-IR spectra of chitin and chitin nanocrystals prepared by acidic DESs.

3.3 Morphology of ChNCs

The morphology of the ChNCs based on AFM analysis is shown in **Figure 3**, and the corresponding dimensions are summarized in **Table 2** from analysis of >20 individual nanocrystals in each sample. The diameter of chitin nanocrystals did not show significant difference and ranged from 29 to 83 nm. However, the length of nanocrystals varied (165-844 nm), OA-ChNC3 and DA-ChNC3 showed a longer length in comparison of other samples. The length of MA-ChNC1 was the shortest among samples, indicating that malonic acid penetrates easily into amorphous area of chitin and promotes the hydrolysis reaction. The shape of ChNCs also altered, and LA-ChNC1 and CA-ChNC1 contained mostly individual needle-like entities while other samples consisted mainly of larger nanocrystal bundles. Previous studies have shown that LA dissolves efficiently the amorphous sections of chitin molecule despite it had the highest pK_a (3.86) value among the used acids (Featherstone & Rodgers, 1981; Qi et al., 2013). The chitin with

12

244   diversity dimensions can potentially be applied in different areas. For example, as dimension of

245   ChNCs can affect significantly the properties of composites, and the ChNCs with a higher aspect

246   ratio are preferred when used as reinforcement in polymer matrix (Gopalan Nair & Dufresne,

247   2003). The ChNCs can also be used as green and sustainable emulsifiers for o/w Pickering

248   emulsions (Tzoumaki, Moschakis, Kiosseoglou & Biliaderis, 2011). The stability of emulsion can

249   be tuned by using different aspect ratios of nano chitin (Bai et al., 2019).
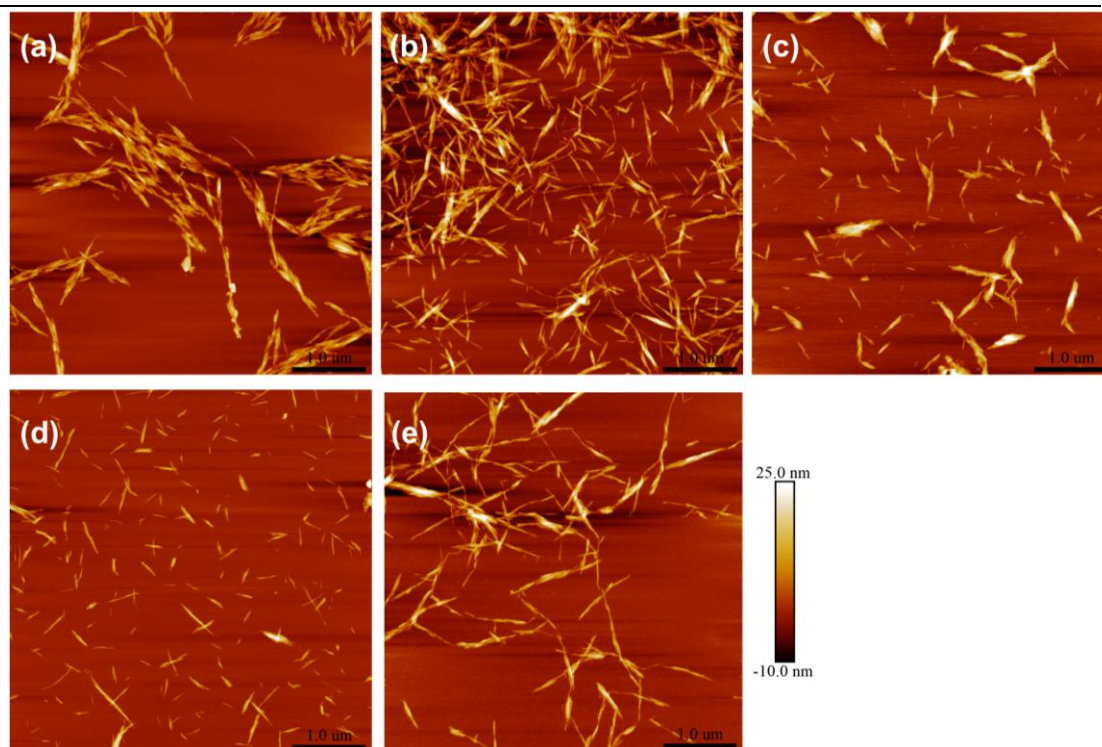
250   Production of nano chitin has been reported through different strategies. Choline chloride and

251   thiourea was used for dissolving chitin first (Mukesh et al., 2014), and the addition of water could

252   regenerate the chitin from the solvent. After sonication treatment of regenerated chitin in water,

253   chitin nanofibers of diameter 20 ± 6 nm and length 1 ± 0.5 μm were obtained with yield up to 84%.

254   Individualized ChNCs were produced by introducing O-acetylation group on the surface of chitin

255   (Hong et al., 2019b). In this treatment DES of choline chloride and zinc chloride was used as

256   nonvolatile solvent and catalysis for the O-acetylation of chitin with the existence of acetic

257   anhydride or acetic acid. During the O-acetylation reaction, hydrolysis of chitin proceeded

258   simultaneously. After sonication treatment, ChNCs with diameter ranging from 20 to 80 nm and

259   length ranged from 100 to 700 nm with yield up to 73.8% were obtained. Moreover, an acidic

260   DES composed with choline chloride and p-toluenesulfonic acid was also used to hydrolysis of

261   chitin for production of ChNCs. The average diameter and length of resulted ChNCs were 26.6 nm

262   and 293.4 nm, respectively. The dimensions of the nano chitin were quite similar as in the current

263   study. However, the appearance of ChNC suspensions in the previous studies was milky like due

264   to the aggregation of ChNC. Moreover, the yield of ChNCs (LA-ChNC1) in this study is higher

265   than previously reported. Based on above results the possible mechanism of DES for producing

Table 2:

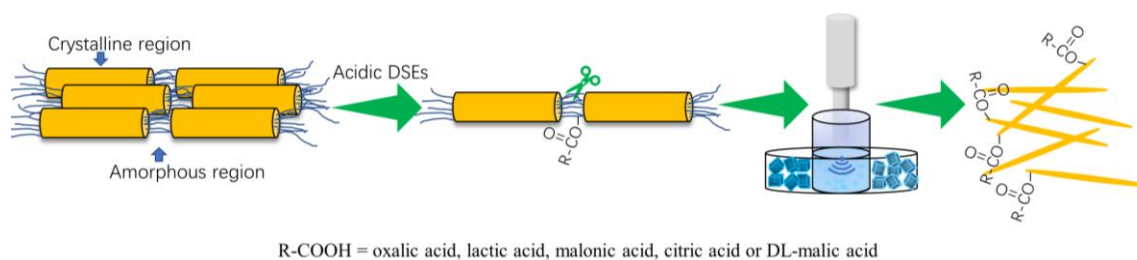Dimensions of chitin nanocrystals.

Sample	Length (nm)	Diameter (nm)	Aspect ratio
OA-ChNC3	530±89	49±8	11
LA-ChNC1	367±48	44±11	8
MA-ChNC1	257±59	47±10	5
CA-ChNC1	306±58	42±10	7
DA-ChNC3	670±92	49±10	14

Figure 3. AFM images of chitin nanocrystals prepared by (a) choline chloride/oxalic acid dihydrate, (b) choline chloride/lactic acid, (c) choline chloride/malonic acid, (d) choline chloride/citric acid monohydrate and (e) choline chloride/ DL-malic acid.
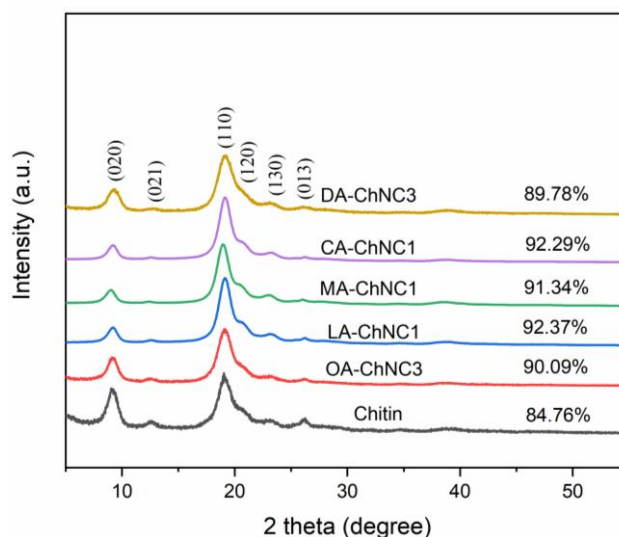

R-COOH = oxalic acid, lactic acid, malonic acid, citric acid or DL-malic acid

Figure 4. Mechanistic illustration for producing ChNCs in organic acid DESs.

3.4 Crystalline structure of ChNCs

The XRD patterns of the pristine chitin powder and lyophilized DESs chitin nanocrystals are

277   shown in **Figure 5**. The XRD was used to examine the effects of different acidic DESs' treatments

278   on crystallinity of chitin nanocrystals. The diffraction peaks at 2θ of 9.2°, 12.5°, 19.2°, 20.6°,

279   23.2°, and 26.3°, and correspond to the diffraction planes of (020), (021), (110), (120), (130), and

280   (013), respectively. All of these patterns are the typical antiparallel crystalline patterns of α-chitin

281   (Hong et al., 2018; Jang, Kong, Jeong, Lee & Nah, 2004; Kaya et al., 2017). The crystalline

282   structure of chitin nanocrystals is in good agreement with the pristine chitin indicating that the

283   crystalline structure of the ChNCs remained unchanged during the preparation process (DESs

284   treatment and ultrasonication) (Jang et al., 2004).

285   After acidic DESs treatment, the CrI values of the obtained chitin nanocrystals increased as

286   expected due to the removal and dissolution of amorphous parts of chitin. This result is different

287   with our previous study in which O-acetylated chitin nanocrystals were prepared. The introduced

288   acetyl groups were found to weaken the hydrogen bonding interactions in chitin and decrease the

289   CrI value (Hong et al., 2019b). Also the CrI value of malate chitin was noted to be lower than that

290   of the pristine chitin (Feng et al., 2019). The CrI value of LA-ChNC1 (92.37%), MA-ChNC1

291   (91.34%), and CA-ChNC1 (92.29%) was slightly higher than that of OA-ChNC3 (90.09%) and

292   DA-ChNC3 (89.78%). This result is in accordance with the micromorphology of chitin

293   nanocrystals obtained with AFM (Figure 3). The length of OA-ChNC3 and DA-ChNC3 was

294   shorter than the other three, reflecting the milder reaction conditions and lower degree of
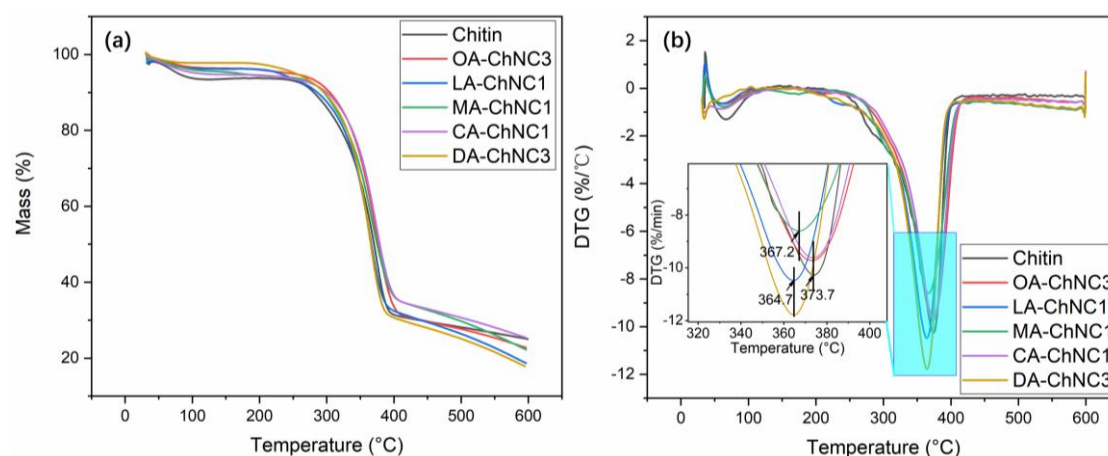
295   dissolution of amorphous chitin.

Figure 5. XRD diffraction patterns of chitin and chitin nanocrystals prepared by acidic DESs.

3.5 Thermal properties of ChNCs

The thermal stability of chitin nanocrystals and pristine chitin were investigated by thermogravimetric analysis. The results of the TGA and DTG curves are shown in **Figure 6**. It can be seen from the TGA curve that the weight loss of the sample is mainly manifested in two stages. The weight loss in the first stage before 200 °C is due to evaporation of water (Kaya, Sofi, Sargin & Mujtaba, 2016). At this stage, the weight loss of OA-ChNC3, LA-ChNC1, MA-ChNC1, CA-ChNC1, DA-ChNC3 and pristine chitin was 4.0%, 3.7%, 3.9%, 5.6%, 5.4% and 5.5%, respectively. No notable differences were observed during this stage. The weight loss in the second stage was between 200 °C and 400 °C, which is due to the saccharide ring dehydration and deterioration of acetylated chitin units (Paulino, Simionato, Garcia & Nozaki, 2006). The total weight loss of OA-ChNC3, LA-ChNC1, MA-ChNC1, CA-ChNC1, DA-ChNC3 and pristine chitin was 64.5%, 62.7%, 63.6%, 58.0%, 58.0% and 63.8%, respectively. Acidic DESs treated chitin showed a less weight loss (%) during this stage, the small difference is most likely due to the O-acylated chitin had fewer free hydroxy groups for dehydration.

The temperature at the maximum (T_{max}) degradation rate of each sample can be seen from the

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313　DTG curve, which were 373.7 °C for pristine chitin, OA-ChNC3 and CA-ChNC1, 367.2 °C for

314　MA-ChNC1, 364.7 °C for L A-ChNC1 and DA-ChNC3, respectively. Many factors could affect

315　the thermal stability of chitin, e.g. molecule weight, degree of crystallinity, particle size and

316　introduced groups on chitin, et al (Hong et al., 2019a; Hong et al., 2019b; Hong et al., 2018;

317　Sajomsang & Gonil, 2010). The thermal stability of OA-ChNC3 and CA-ChNC1 is similar to that

318　of pristine chitin while LA-ChNC1, DA-ChNC3 and DA-ChNC3 showed a lower stability. In melt

319　compounding, natural nanoparticles such as chitin and cellulose nanocrystals, can be used as

320　environmentally friendly reinforcements. The processing temperature for thermoplastics can be up

321　to 200 °C (Zhao, Du, Chen, Pan & Chen, 2019) and the thermal stable chitin nanocrystals are

322　potential green materials in these applications. Therefore, thermal stable chitin nanocrystals

323　prepared in this study can be seen as potential reinforcements in thermoplastics.

324
325
326　**Figure 6.** TGA and DTG curves of chitin and chitin nanocrystals prepared by acidic DESs.

327　**4. Conclusion**

328　　The acidic DESs composed of choline chloride and the oxalic acid dihydrate, lactic acid,

329　malonic acid, citric acid monohydrate and DL-malic acid were successfully used to production of

330　chitin nanocrystals at 100 ° C. The properties of ChNCs were found to be affected by the reaction

331   time of DES treatment. The physicochemical properties of ChNCs were compared and studied

332   through FTIR, AFM, XRD and TGA. All chitin nanocrystals were found to be O-acylated. The

333   average diameter of individual chitin nanocrystals was in range of 42 nm to 49 nm with average

334   length of 257 - 670 nm. After acidic DESs treatment, the removal of amorphous area led to the

335   increase of degree of crystallinity of chitin nanocrystals. The thermal stability of all chitin

336   nanocrystals was comparable to that of pristine one. Among them, DES of choline chloride and

337   lactic acid was considered as the optimal choice due to a shorter reaction time, the highest mass

338   yield of ChNCs (up to 87.5 wt%), and stable aqueous suspension. The possible application of this

339   kind stable suspensions can be emulsifiers for Pickering emulsions. Thus, the acidic DES-systems

340   proved to be simple, effective and environmentally friendly approaches to prepare O-acylation

341   chitin nanocrystals in one step reaction.

347

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