



Urea as a single denaturing agent in deproteinisation of natural rubber latex

A. Nurulhuda¹ · A. H. Aziana¹ · A. R. Norazreen¹ · A. Nur Aziah¹ · M. Siti Nor Qamarina¹

Received: 15 February 2019 / Accepted: 31 May 2019
© The Malaysian Rubber Board 2019

Abstract

This paper describes the utilisation of urea as a single chemical agent to obtain deproteinised high ammoniated natural rubber latex (HA-NRL). The role of urea in HA-NRL deproteinisation was further investigated by employing two different concentrations of urea solution which are low (0.01–0.05%) and high (1.0–5.0%) concentrations. The study assessed the potential usage of urea as a single denaturing agent in the presence of neither stabilising agent nor additives during the deproteinisation process. The effect of different urea concentrations on properties of deproteinised NRL was further scrutinised by using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), nitrogen content analysis, extractable protein (EP) content, Zeta potential, attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy and physical strength. The presence of residual urea is pertinent as the concentration of urea increases while contributing to the increase of total nitrogen content in the resultant film. Leaching the film with hot water is sufficient to remove the residual urea as supported by an ATR-FTIR analysis. Initial exploratory data analysis of low and high urea concentrations on the effect of nitrogen content, EP, surface charge properties of latex and green strength of the deproteinised NRL were evaluated and their relation to each factor was further discussed. Based on the initial findings, urea was observed to be less effective as a single denaturant. Nonetheless, the properties of the deproteinised HA-NRL treated with urea were revealed to be markedly influenced by the urea concentration.

Keywords Deproteinised · Natural rubber latex · Urea · Protein

Introduction

Natural rubber latex (NRL) has been used as a base material for the production of gloves for more than a century [1]. In the 1980s and 1990s, the use of protective gloves in health-care was increased dramatically due to the implementation of the so-called ‘universal precautions’ by the Centres for Disease Control in the US (1987) to minimise the risks of HIV and hepatitis infection [2]. Although NRL films offer excellent properties especially in medical field applications, however, the presence of proteins particularly the extractable proteins in various NRL products have been identified to cause allergies within specific risk groups which include health care workers, latex industry manufacturing workers, spina bifida patients, and other sensitised populations. In the

latex glove industry, total extractable proteins from gloves are used as an indicator of their potential allergenicity. Following that, allergic reactions toward proteins in NRL products has emerged as one of the most significant occupational diseases among healthcare workers, of which the first few cases were reported in Europe and later in the United States.

The total protein content of fresh latex is in the region of 2% in total compared to the other components in natural rubber latex. Of this 2%, almost 20% comprises proteins that are adsorbed on the rubber particles, while a comparable percentage is related to the bottom fraction, and the remainder is present as part of the dissolved proteins in the serum phase. Proteins in NRL can also be distributed into two distinct fractions, namely the rubber and serum fractions. A major component of the adsorbed proteins on the rubber particles is presumably α -globulin which is insoluble in pure water [3]. Hevein, the second major protein is water soluble, it is present abundantly in the bottom fraction of latex. However, specific studies on rubber particles’ protein adsorption are scarce due to the difficulty

✉ A. Nurulhuda
nurulhuda.a@lgm.gov.my

¹ Malaysian Rubber Board, RRIM Research Station,
47000 Sungai Buloh, Selangor, Malaysia

of removing the adsorbed proteins without changing their native structure from the surface of rubber particles.

Urea is a small organic molecule that is highly soluble in water over various ranges of concentrations and integrates itself well in the hydrogen-bond network of water. Urea is known as one of the chemicals that can change the protein conformation and hence subsequently denature the proteins, while their interactions have been well established for a long time [4–8]. Urea is among other chemicals that are capable of changing protein conformation due to its possible interactions with proteins in the course of denaturation [9]. Urea can easily denature the surface-bound proteins as it is easier to disengage proteins on the surface of the rubber particle. However, some proteins that are still tightly bound to the rubber particles pose further difficulties to be removed. Besides, it is also suggested that the addition of urea in water can possibly destabilise the folded proteins through a protein binding mechanism and compete with its innate interaction, which actively participates in the unfolding process [10]. Once these proteins are denatured, changes in their structure may occur, resulting in loss of their ability to perform native biological activities [11].

Although urea is a common denaturant used to denature proteins, however, the exact mechanism during urea deproteinisation remains unknown. As reported in the previous study [12], there are actually two basic lines of reasoning proposed for the denaturing effect of urea. Urea may exert its effect directly, by binding to the protein, or indirectly, by altering the solvent environment. In the direct mechanism, it was found that the most favourable interaction of urea is with the peptide backbone of protein and this interaction had resulted in the unfolding process. The direct mechanism focuses mainly on the nature and strength of urea interactions with the polar/apolar side chains and the peptide backbone, and on the role of hydrogen bonding to reduce the hydrophobic effect.

While in the indirect mechanism, it is postulated that urea promotes ready dissolution of hydrophobic groups by altering the water structure. The effect of urea on water structure is attributed to the radial distribution functions, causing a decrease in water–water interactions and hydrogen bond strength. This process allows urea to be readily substituted into the water hydrogen bond network, omitting segregation of urea from the water. It is claimed that the water structure is unchangeable by urea but urea itself may form dimers and oligomers due to the hydrogen bonds between the $-NH$ and $-CO$ groups, similar to the hydrogen-bonded secondary structures found in proteins. Hence, urea has weakened the structure of water and the ordering of water close to hydrophobic surfaces, thereby making water better at accommodating hydrophobic solutes. Generally, the denaturation of urea is attributable to both mechanisms [10, 13].

This paper describes the role of urea as a single denaturant agent in the deproteinisation process of NRL. The effect of different concentrations of urea was assessed to confirm its reactivity as a single protein denaturant while targeting to produce NRL with reduced allergenic risks and potentially higher mechanical strength. On the other hand, a study between two extreme urea concentrations was performed to understand their role in affecting nitrogen content, extractable protein content, Zeta potential, and green strength. This approach could potentially reduce the chemical cost and at the same, optimise the function of urea.

Experimental

Materials

Natural rubber latex concentrate grade, preserved with 0.7 wt% ammonia (HA-NRL) and all chemicals were used as supplied without further purification throughout this study.

Preparation of deproteinised latex

Commercial (HA-NRL) was used for the preparation of deproteinised NRL. HA-NRL was incubated with urea (w/w) at predetermined low concentrations of 0, 0.01, 0.02, 0.03, 0.04 and 0.05% respectively, in a mixing vessel under continuous stirring for 2 h prior to centrifugation. Concurrently, HA-NRL was also incubated with another set of higher concentrations of urea comprising 1.0, 2.0, 3.0, 4.0 and 5.0%, following the exact procedure for low concentration of urea. Centrifugation was carried out using the Beckman Coulter Ultracentrifuge Model Optima L-100K at 9000 rpm and 15 °C for 30 min. After centrifugation, the cream fraction was collected and redispersed with distilled water to make up for 40% total solid content (TSC) and stirred for 30 min. The dispersed latex underwent another centrifugation at 9000 rpm and 15 °C for 30 min. The resulting rubber cream fraction was again collected and resuspended in distilled water to make up for 30% TSC of latex. This centrifugation process will ensure that an optimal purification of deproteinised HA-NRL is achievable.

Characterisation

In the determination of proteins molecular weight procedure, the latex was firstly centrifuged at 21 000 rpm for 1 h ($T = 5\text{ }^{\circ}\text{C}$) to have a clear serum fraction. The sample from serum fraction further diluted with phosphate buffer solution (PBS) and protein loading buffer (1.0 M Tris HCl (pH 8.5), 8% (w/v) lithium dodecyl sulphate, 40% (v/w) glycerol, 2 mM EDTA, 0.5 M DTT and bromophenol blue) before being subjected to SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) which contains 4% stacking gels and 15% separating gels. The SDS-PAGE analysis was conducted at a constant current of 0.25 A for 1 h. The protein molecular weight standard (thermo scientific) was run alongside in all experiments. The SDS-PAGE gels were stained either with Coomassie Blue R-250 or silver staining method afterward before being destained in destaining solution.

An approximate 0.3 mm thick NR as per prepared deproteinised latex films were prepared by casting on level glass plates and left to air-dry at room temperature for 3 days before undergoing further characterisation tests. The appearance and disappearance of some functional groups of resultant cast films before and after the treatments were analysed using the ATR-FTIR technique. The samples were analysed at wavenumbers ranging from 4000 to 200 cm^{-1} . Protein content in the above-mentioned casted film was determined using the Kjeldahl method to measure the total nitrogen content. About 0.1 g of the cast film sample was first weighed and digested in a micro Kjeldahl flask, followed by steam distillation. The distillation result was titrated with 0.01 N standard sulphuric acid. The nitrogen content was determined in triplicates and calculated from the titration values. Total extractable protein (EP) content was determined according to the *ASTM D5712-99* test method. The water-soluble proteins were extracted out from latex film specimens ($6 \times 6 \text{ cm}^2$) by leaching in phosphate-buffered saline (PBS) (1 g/5 mL) under a temperature of $23 \pm 2^\circ \text{C}$ for 2 h. The soluble proteins were precipitated out and then dissolved and quantified based on the development of colour.

Approximately 0.01 wt% of samples were prepared by diluting the latex with distilled water prior to the test. Zeta potential of resultant latex was determined by ZetaPlus (Brookhaven Instruments Corporation) with measurement run at $T = 25^\circ \text{C}$. Each experiment was repeated five times to give an average value of Zeta potential. The pH value of the system was adjusted to pH 10–11 with 0.1 M KOH and pH were recorded using a calibrated pH meter.

The tensile strength of un-aged casted film ($0.3 \pm 0.5 \text{ mm}$ thickness) was performed using an Instron Tensile Machine 5565 with a crosshead speed of 500 mm/min according to the *ISO 37* test method.

Results and discussion

Protein molecular weight profile of HA-NRL after treatment with low and high urea concentration using SDS-PAGE

Molecular weight profiles of proteins present in the serum of NRL treated with different urea concentrations are depicted as shown in Fig. 1a–c. Initially, when the SDS-PAGE was stained with Coomassie Brilliant Blue (not

shown in the figure), no proteins were visibly detected in the serum of HA-NRL treated with low urea concentrations (0.01–0.05%), possibly because the protein content is too diluted. However, using a similar amount of sample, smaller size proteins were weakly detected when SDS-PAGE was stained using a silver method for the serum of NRL that was treated with 0.05% urea. To reconfirm the presence of denatured proteins, the crude serum samples from two different concentrations namely; 0.03% and 0.05%, were re-analysed on the SDS-PAGE using the silver staining method. As the serum sample concentration increases, a clearer protein band separation at a smaller protein band for 0.05% (Fig. 1b) was observed as compared to that of 0.03%. It was worth noting that the HA-NRL control serum exhibited greater band separations, detected at higher protein molecular weight band (Fig. 1a), while serum of HA-NRL treated with 0.05% and 0.03% showed detection at smaller sizes of proteins ranging from 15 to 25 kDa. This trend suggests the efficacy of urea in denaturing large molecular weight proteins in HA-NRL into smaller molecular weight proteins.

Meanwhile, at higher concentrations of urea (1.0–5.0%), staining with Coomassie Brilliant Blue protein method is preferred as it resulted in distinct protein band separations, in comparison to the silver staining procedure. Regardless of staining techniques practiced, it is worth to note that no significant changes of a protein profile in HA-NRL serum were observed in the presence or absence of urea. Apparently, a similar protein profile was spotted between control (untreated HA-NRL) and HA-NRL treated with different concentrations of urea (1.0–5.0%). Although protein bands were not well-separated, a few bands of protein were noticed at sizes between 25 and 40 kDa. The smearing condition could be attributed to the presence of impurities or polypeptides residual caused by the denaturation process mediated by the high concentration of urea, hence inhibits the protein molecules to pass through the polyacrylamide gel. Meanwhile, the high MW protein molecules could be originated from disentanglement of protein polypeptide chains by urea. This polypeptide chains probably were not fully denatured because it is also known that the concentration of urea that is needed to denature a protein, depends on the type of protein [12]. Hence, to obtain an optimum concentration of urea as a single denaturant, we need to further identify the protein and peptide content of NRL.

Nitrogen and extractable protein content

Figure 2 illustrates the total nitrogen content and extractable protein (EP) content of HA-NRL treated with different concentrations of urea. Nitrogen content analysis is a method that is widely used to analyse and determine protein content in a sample. A conversion factor of 6.25, which is equivalent

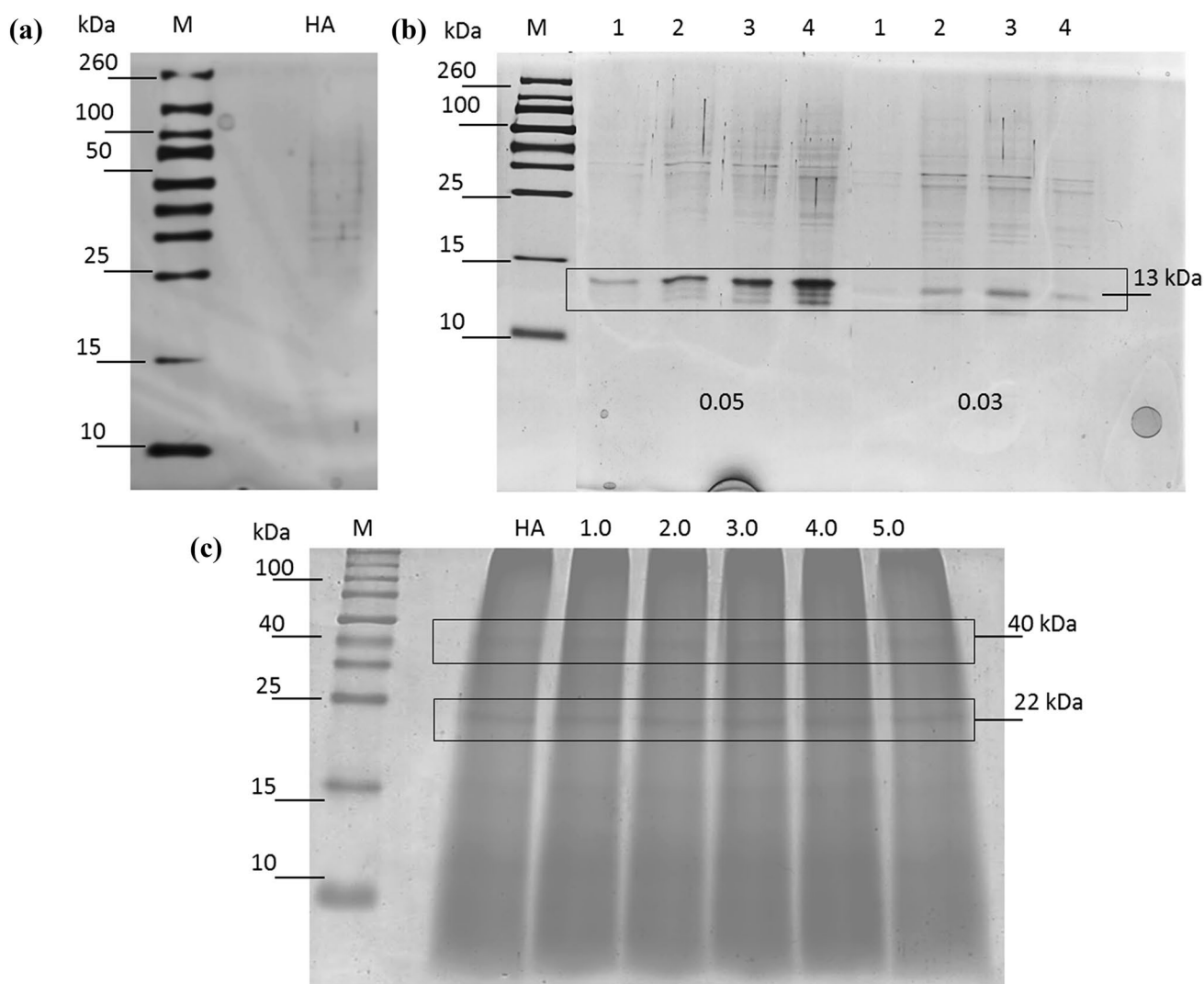


Fig. 1 Analysis of SDS-PAGE on the serum of HA-NRL treated with different concentrations of urea; **a** HA-NRL (control) and **b** selectively at 0.03% and 0.05% urea concentration, 1 to 4 indicates the amount of sample loaded on the SDS-PAGE; 10 µl, 20 µl, 30 µl, and 40 µl with 2 µl protein loading buffer, respectively **c** high concentra-

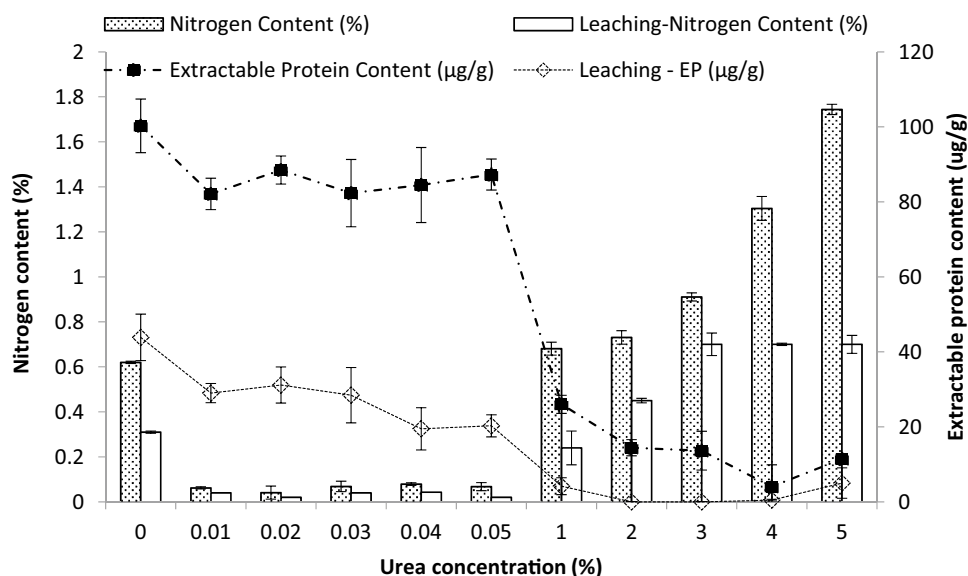
tion of urea 1.0 to 5.0%. Gel **a** and **b** was stained with the silver staining method while gel **c** was stained with Commassie Brilliant Blue method. Lane M represents the protein molecular weight standard with their respective sizes in kDa

to 0.16 g of nitrogen per gram protein, is used to calculate the protein content.

As described in the graph, an obvious escalation in total nitrogen content was observed to follow a linear trend as the concentration of urea employed in the HA-NRL increases. This trend is presumed to be due to the presence of residual urea that contributes to the increments in the nitrogen content as the Kjeldahl method measured the total amount of nitrogen present in the latex system [14]. Another contributing factor of high nitrogen content would be attributed to the absence of surfactant in this system. The absence of a surfactant may suppress the effectiveness of urea in the process of protein denaturation and impeded removal of residual urea during centrifugation.

Clearly, lowering urea concentrations did not give a marked difference in their nitrogen and EP content collectively. Contrarily, as the urea concentration increased, the EP content significantly decreased. Two distinctive regions were shown in the graph for both extreme concentrations. A sharp decreasing trend between 0.05% and 1.0% of urea concentration on EP content was observed giving about 70% reduction from 0.05% urea concentration to 1.0% urea concentration. EP content is used to measure the readily solubilised protein from the HA-NRL film. The analysis of nitrogen and EP content was performed using the same film sheet from a casting procedure. Although high urea concentrations facilitated protein solubilisation, its utilisation at a higher concentrations for NRL deproteinisation is deemed unnecessary. On

Fig. 2 Analysis of total Nitrogen content and extractable protein content of leached and unleached HA-NRL films treated with different urea concentrations



the other hand, there is a significant reduction observed in the nitrogen and the EP content which is due to the effect of the hot water leaching process. It is a good combination to implement leaching protocols together with urea-HANRL deproteinisation to reduce further the EP content.

Based on the EP analysis, the trend indicated that 1.0–2.0% may be an optimum urea concentration for HA-NRL deproteinisation as it gives a maximum reduction in their EP content albeit the high nitrogen content confirmed via the Kjeldahl method; which is presumably due to the presence of residual urea. Subsequent analysis was performed to support this hypothesis. It is also suggested in another study [10] that the mechanism for urea-unfolding-protein may be governed by urea concentration. At a low concentration regime, it is proposed that urea exerts its effect indirectly, by altering the solvent environment. However, at higher concentrations, urea may interact directly and indirectly to unfold and denature the protein. It is suggested that in high urea concentrations, urea will bind to the protein, resulting in expansion of the hydrophobic core of the protein due to water penetration. It is anticipated that intricate urea–protein interactions at high urea concentrations might govern these findings.

ATR-FTIR analysis

ATR-FTIR spectroscopy of low (Fig. 3a) and high concentration (Fig. 3b) urea from treated HA-NRL cast films are shown, respectively. The results illustrate the presence of the amines functional group in both treatments; confirmed by the stretching of N–H at region I (3400–3000 cm^{-1}) and stretching of C–N at region II (1126–1150 cm^{-1}). In Fig. 3a, it is noticeable that as the concentration of urea increases, band broadening has become apparent at region I, which

indicated an accumulation of urea residue in the resultant film. On the other hand, Fig. 3b confirms that the presence of residual urea as a band at region I are splitting, forming distinctive peaks which intensified as the concentration of urea increases up to 5.0%. This observation supports the assumption that the high nitrogen content analysis as a result of the Kjeldahl method is attributed to the presence of residual urea.

The effect of leaching protocol (70 °C/water/2 min) onto the resultant film had evidently diminished the band at the predetermined region I, which associates with the presence of urea residual (characterised by the dotted line). It is, therefore, evident that nitrogen content determined from the Kjeldahl analysis is not proportional to that of protein content as shown in Fig. 2 since the nitrogen element from residual urea is also determined.

Zeta potential analysis of urea treated HA-NRL

Table 1 below describes the effect of different urea concentration treatments towards Zeta potential measurement of the resultant latex. Zeta potential analysis is a direct measurement used to determine colloidal stability of particles by quantifying the surface charges of the particle. Rubber latex has been claimed and reported elsewhere, to maintain its Zeta potential range between –30 mV and +30 mV [15]. It was recently found that the hydrophobic core of rubber molecules are surrounded by a mixed layer of proteins and phospholipids and these constituents are found to be phase separated into distinct domains within that layer [16]. As such, the stability of HA-NRL is actually rendered by the presence of adsorbed proteins on the surface of rubber latex, hence resulting in higher Zeta potential.

Fig. 3 ATR-FTIR analysis of **a** low concentration of urea treated HA-NRL film and **b** high concentration of urea treated HA-NRL film. L refers to the leaching process

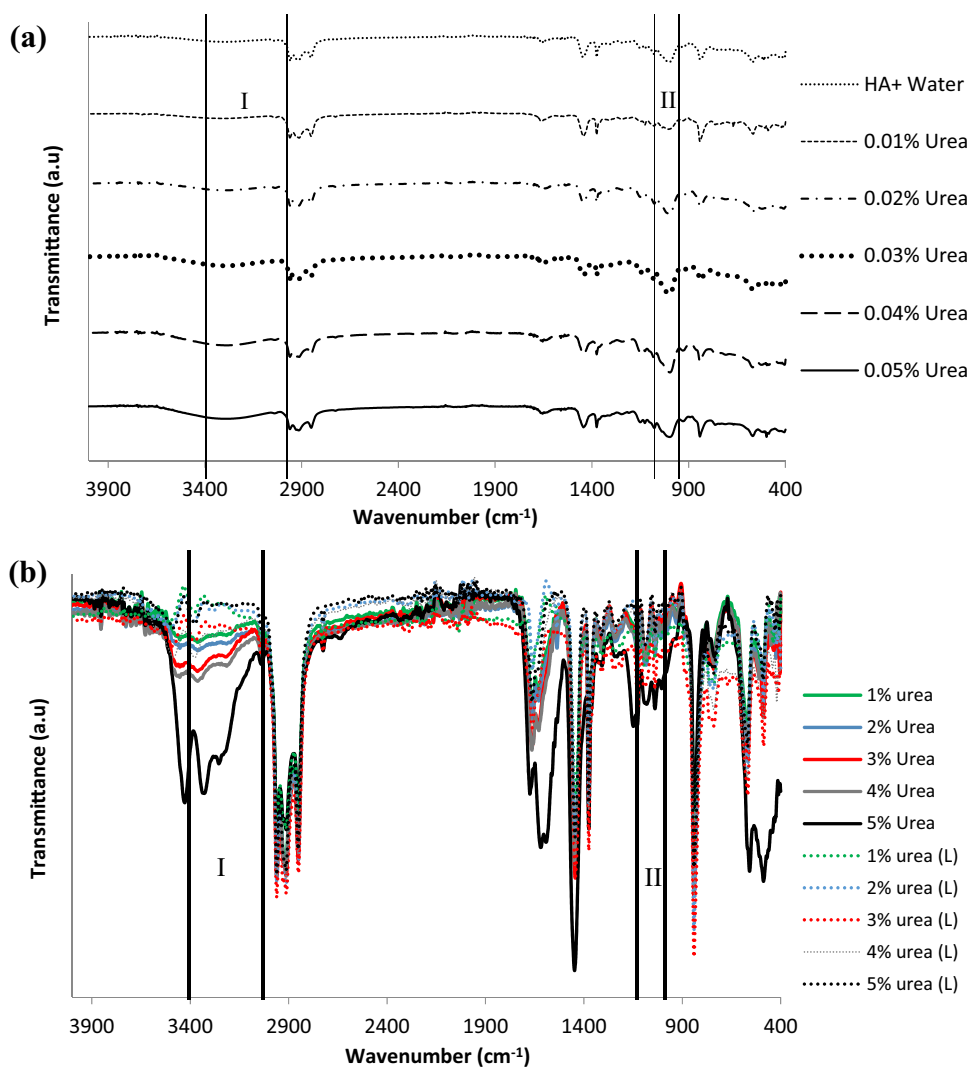


Table 1 Zeta potential analysis of HA-NRL latex treated with different concentration of urea determined at pH 10–11

Sample	Treatments	Zeta potential (mV) ^a	Zeta potential (mV) ^b
A	HA-NRL + water	-69.8 ± 1.1	-61.7 ± 2.9
B	HA-NRL + 0.01% urea	-61.1 ± 1.8	-53.4 ± 1.2
C	HA-NRL + 0.02% urea	-69.4 ± 2.6	-50.7 ± 1.1
D	HA-NRL + 0.03% urea	-68.4 ± 1.5	-35.3 ± 2.1
E	HA-NRL + 0.04% urea	-55.5 ± 1.8	-39.1 ± 2.0
F	HA-NRL + 0.05% urea	-68.1 ± 1.7	-42.3 ± 3.1
G	HA-NRL + 1% urea	-40.6 ± 1.1	-31.7 ± 2.8
H	HA-NRL + 2% urea	-33.6 ± 2.8	-41.5 ± 1.1
I	HA-NRL + 3% urea	-40.6 ± 1.6	-29.5 ± 2.8
J	HA-NRL + 4% urea	-34.2 ± 2.9	-35.1 ± 2.0
K	HA-NRL + 5% urea	-39.9 ± 1.5	-30.2 ± 3.1

^aMeasurement is taken on day 1 after production day

^bMeasurement is taken on day 5 after production day

It was evident that the deproteinisation of HA-NRL by urea affected the surface charge distribution of latex during the treatment. A noticeable reduction was observed in the negative Zeta potential value when the concentration of urea increased, implying the interaction of urea to the latex protein in promoting deproteinisation. Although the Zeta potential of rubber particles reduced, the stability of resultant latex was maintained without self-coagulation for a shorter period. After 5 days, the Zeta potential values decreased revealing the changes in surface charge behaviour over storage.

Effect of urea deproteinisation of HA-NRL on physical strength

The physical strength of unvulcanised urea deproteinised NRL films is depicted in Fig. 4. Interestingly, although the latex was treated with urea in the absence of a stabiliser (e.g. surfactant), its physical strength was found to be comparable with that of the control HA-NRL in a lower urea concentration region. As the concentration of urea increased beyond 1.0%, escalation in physical strength value was recorded. This result infers the probability of the residual urea that is present during the deproteinisation process which had indirectly contributed to a physical strength increment in the unvulcanised latex films.

Initial exploratory data analysis for effect of nitrogen content in urea deproteinisation HA-NRL

Figure 5a–c depicted the effect of nitrogen content on different variable parameters including extractable protein (EP) content, Zeta potential analysis and the physical strength of the unvulcanised films. Evidently, the EP content of the resultant film reduced as the nitrogen content increases

(Fig. 5a). This finding implied that urea serves as protein denaturation agent and assists in producing a lesser amount of EP as the concentration of urea increased. Nevertheless, it is worth to notify that a high amount of nitrogen content might be contributed from the urea molecules or residual urea during deproteinisation. Clearly, this high amount of nitrogen content does not entirely correspond to the protein concentration in the sample.

Adsorbed proteins on the surface of rubber particles are reported to contain both acidic and alkaline functional groups that carry positive and negative charges, respectively [17]. As illustrated in Fig. 5b, the Zeta potential plot tends to shift toward a positive region as the nitrogen concentration increases. This shifting behaviour may be associated with perturbation of the protein layer by urea and as a result, compensate the surface charge. This activity would compress the diffused layer and subsequently reduce the Zeta potential value. From another perspective, the presence of residual urea was anticipated to create an imbalance between two counteracting forces; Van der Waals and the electrical double layer which triggered distribution of the surface charges of rubber particles.

Meanwhile, from the scatter plot of nitrogen content towards physical strength, it was found that tensile strength increases as nitrogen content increases (Fig. 5c). Blackley [18] reported that tensile strength of the resultant latex films is reliant on the ability of the particles to coalesce and integrate when the film dries, and on the concentration of crosslinks in the vulcanised rubber. Hence, the physical strength of these unvulcanised films may be attributed to the physical entanglement of the rubber coil and particle coalescence since no chemical linkage was present between the rubber hydrocarbon bonds.

It is also suggested in the previous study [19] that the physical strength of unvulcanised NR is composed of

Fig. 4 Tensile strength of unvulcanised urea treated HA-NRL

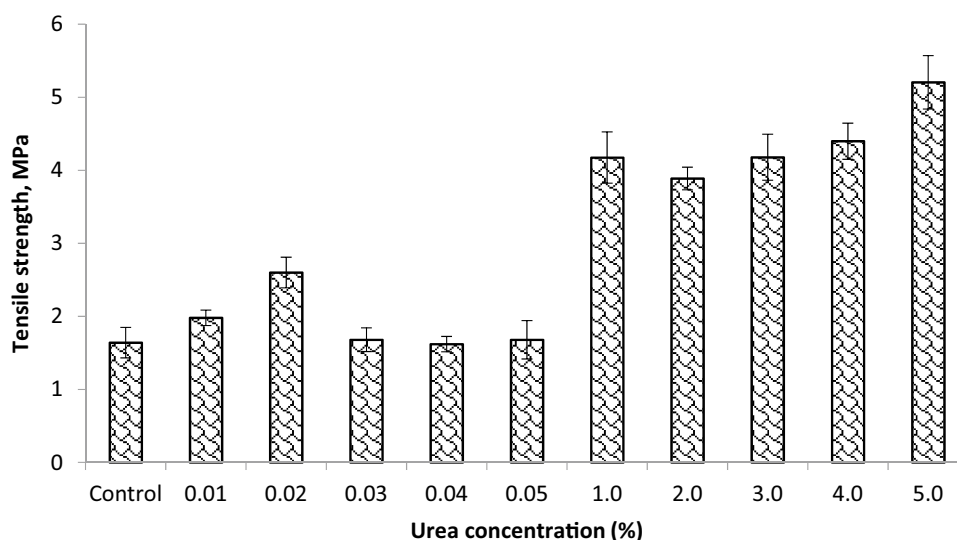
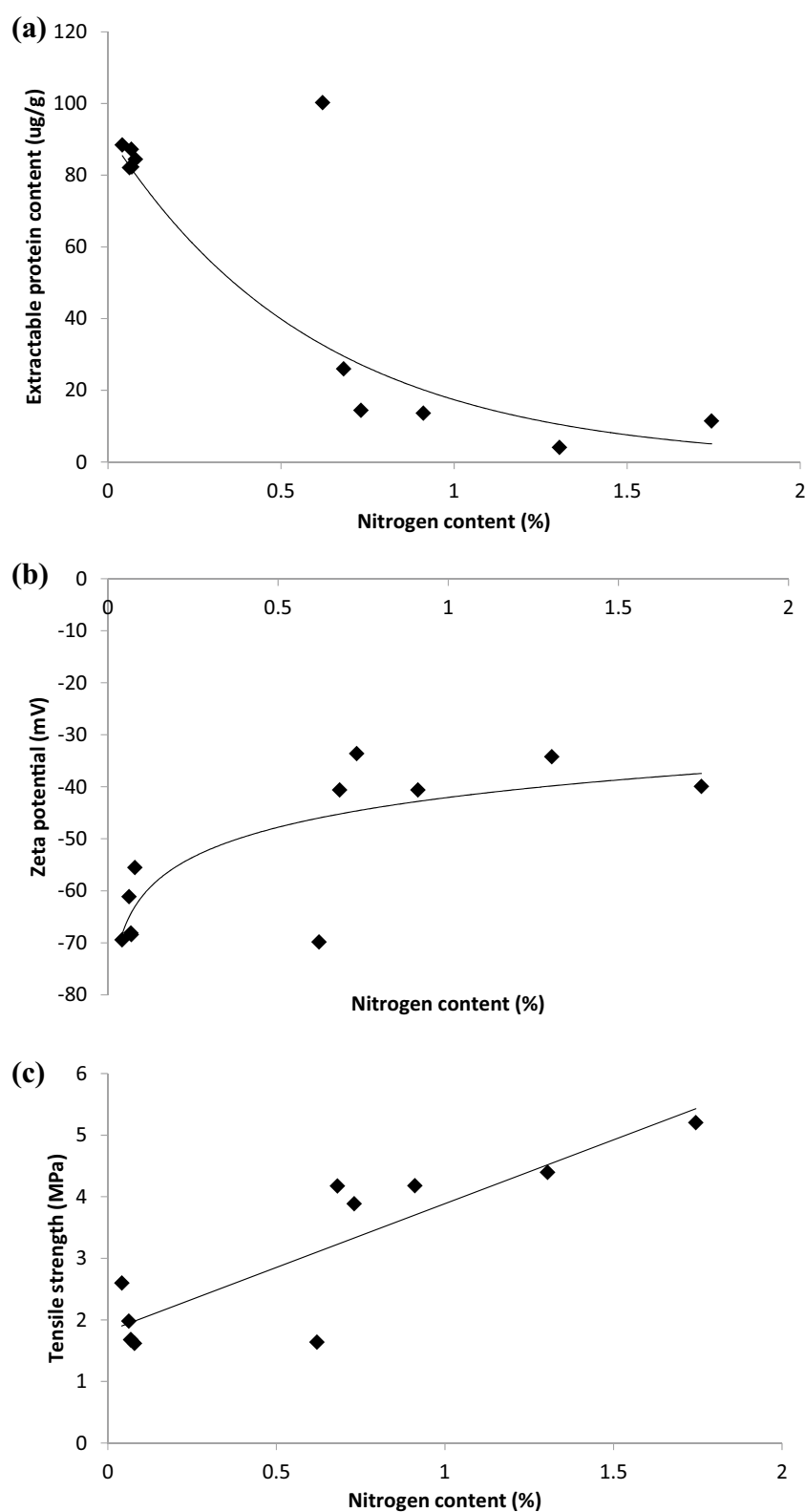


Fig. 5 Scatter plot for effect of nitrogen content onto **a** extractable protein content, **b** Zeta potential and **c** tensile strength in urea deproteinisation of HA-NRL



entanglements, pseudo-end-linked networks and stress-induced crystallisation (SIC). Although the network density of the pseudo-end-linked network is very low, the whole entanglement seemed to become a permanent

entanglement that is not loosened by chain movements. It is reported that during deformation, unvulcanised NR behaves such as rubber with an end-linked network and entanglements.

Conclusion

On the whole, urea is clearly showing its function as a protein unfolding agent. It was observed that a lower urea concentration created a tremendous reduction in the nitrogen content, while at higher urea concentrations, both nitrogen and EP contents from NRL cast films experienced a significant reduction. It is assumed that at a low concentration of urea, an indirect protein unfolding mechanism occurs which involved solvation of urea into the hydrophobic core of proteins; hence, urea was effectively used during the reaction. However, as the urea concentration is increased, a stronger interaction of urea with the peptide backbone of proteins may occur resulting in the accumulation of residual urea on the rubber particles which is difficult to be removed. It is suggested that urea concentration is maintained between the range of 1.0–2.0% as an optimum urea concentration for the NRL deproteinisation process as beyond that, residual urea will have detrimental effects on the NRL film. Since in this study, no surfactant was used to facilitate urea protein removal, it was perceived that even at higher urea concentrations, the eradication of unfolded proteins from the deproteinised NRL will be ineffective. Hence, it is crucial to denote that urea is not efficient as a single denaturing agent in HA-NRL deproteinisation systems, in regard to maintaining the colloidal stability of the latex. From this study, it was also noticed that nitrogen analysis content of urea deproteinised NRL may give a false indication of protein content as it analyses the total amount of nitrogen which may be derived from the residual urea. Therefore, a proper leaching protocol allowing for greater proteins and residual urea removal shall be implemented.

Acknowledgements The authors would like to thank the Director General of the Malaysian Rubber Board for permission to publish the findings of this study. The assistance rendered by Ahmad Shawal Razlan, Zuliza Ahmad, Ahmad Syaheer Abu Aswad, and Syamimi Izzati Mohd Asri is highly acknowledged. Technical and scientific English editing by respective editors and reviewers are most appreciated.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Palosuo T (2014) Chemistry, manufacture and applications of natural rubber national institute for health and welfare. Woodhead Publishing Limited, Finland, pp 452–482
- Sussman GL, Beezhold DH, Kurup VP (2002) Allergens and natural rubber proteins. *J Allergy Clin Immunol* 110(2):s33–s39
- Sansatsadeekul Jitlada, Sakdapipanich Jitlada, Rojruthai Pornpip (2011) Characterization of associated proteins and phospholipids in natural rubber latex. *J Biosci Bioeng* 111(6):628–634
- Kawahara Y, Klinklai S, Kuroda W, Isono H (2004) Removal of proteins from natural rubber with urea”. *Polym Adv Technol* 15(4):181–184
- Creighton TE (1984) *Proteins: structures and molecular principles in protein structure determination*, suelter, ch edn. Wiley, New York, p 514
- Greene RF, Pace CN (1974) Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, zymotrypsin, and lactoglobulin. *J Biol Chem* 249(17):5388–5393
- Pace CN (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol* 131:266–280
- Kauzmann W (1959) Some factors in the interpretation of protein denaturation. *Adv Protein Chem* 14:1–63
- Yamamoto PT, Kawahara Y, Chaikumpollert S, Nghia O (2013) Protein-free natural rubber, latex thereof, and method for manufacturing said rubber. US patent no. 8476348
- Bennion BJ, Daggett V (2003) The molecular basis for the chemical denaturation of proteins by urea. *pnas* 100(9):5142–5147
- Creighton TE (1995) Protein folding: an unfolding story. *Curr Biol* 5(4):353–356
- Lindgren M (2010) On the mechanism of urea-induced protein denaturation master dissertation. University Of Umea, Sweden
- Creighton TE (1979) Electrophoretic analysis of the unfolding of proteins by urea. *J Mol Biol* 129(2):235–264
- Yamamoto Y, Nghia PT, Klinklai W, Saito T, Kawahara S (2008) Removal of proteins from natural rubber with urea and its application to continuous processes. *J Appl Polym Sci* 107:2329–2332
- Ariyawiriyana W, Nuinua J, Sae-Hengb K, Kawahara S (2013) The mechanical properties of vulcanized deproteinized natural rubber. *Energy Proced* 34:728–733
- Nawamawat K, Sakdapipanich JT, Chee CH, Ma Y, Song J, Vancso JG (2011) Surface nanostructure of *Hevea brasiliensis* natural rubber latex particles. *Colloids Surf A Physicochem Eng Aspects* 390:157–166
- Kemp AR, Straitiff WG (1940) Hevea latex: effect of proteins and electrolytes on colloidal behavior. *J Phys Chem* 44(6):788–800
- Blackley DC (1997) Chemically-modified lattices: 1. Prevulcanized lattices, chap. 13, polymer latices science and technology, vol. 2: type of lattices, 2nd edn. Chapman and Hall, London
- Shigeyuki T, Che J, Rong L, Hsiao BS, Amnuayporn Sri S, Adul Nimpaboon, Sakdapipanich J (2013) Entanglements and networks to strain-induced crystallization and stress-strain relations in natural rubber and synthetic polyisoprene at various temperatures. *Macromolecules* 46:5238–5248

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.