

Figure 3 TEM of (a) cyan, (b) magenta, (c) yellow and (d) black pigment dispersions; TEM for (e) cyan and (g) yellow inks; and SEM for (f) magenta and (h) black inks after one-year storage

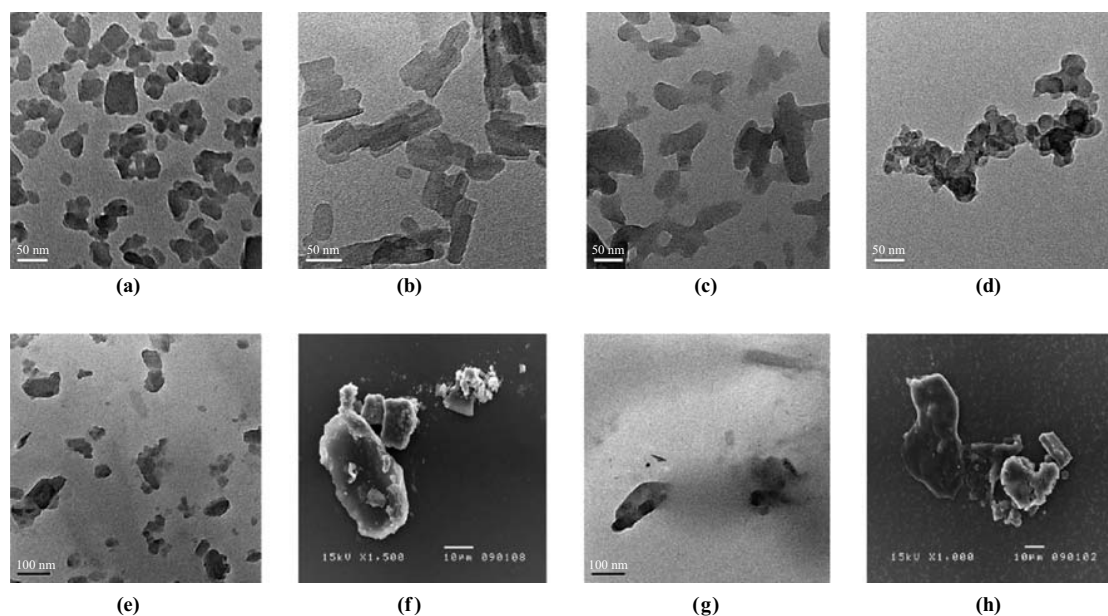
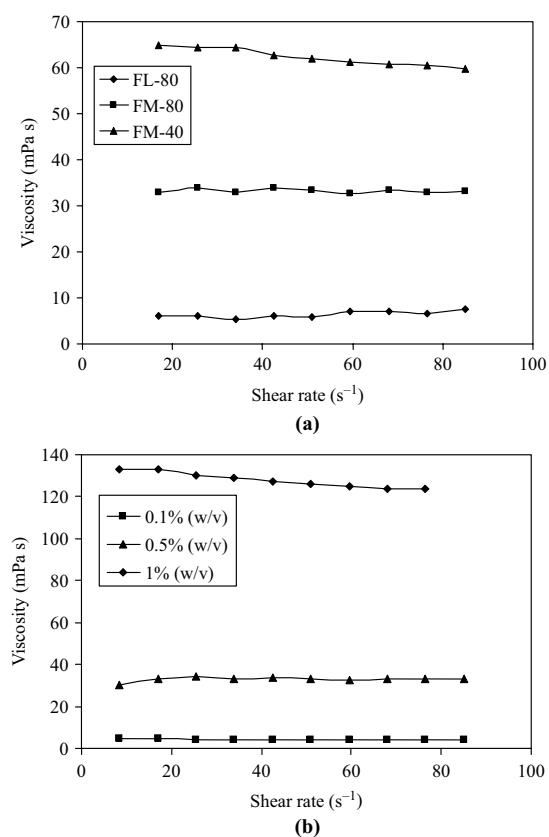


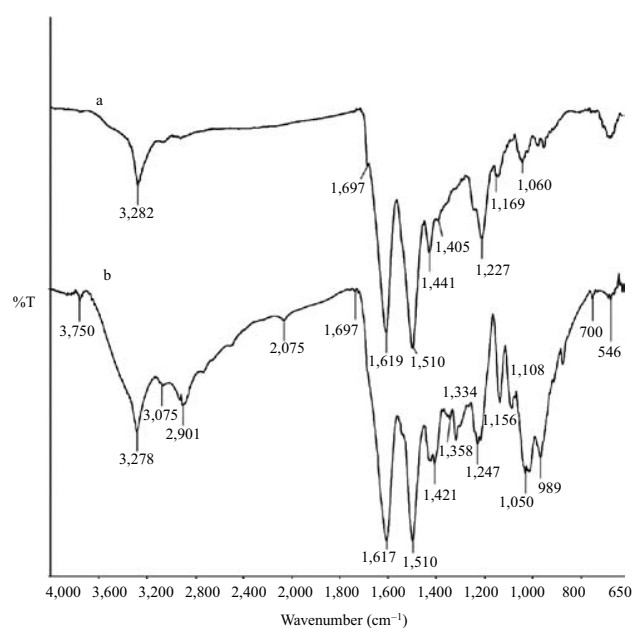
Figure 4 Viscosity of the chitosan solutions



Notes: (a) Types of chitosan used at 0.5 per cent w/v and (b) FM-80 at various loadings at 25°C; data are shown as the mean $\pm$ 1 (SD) and are derived from three replicates

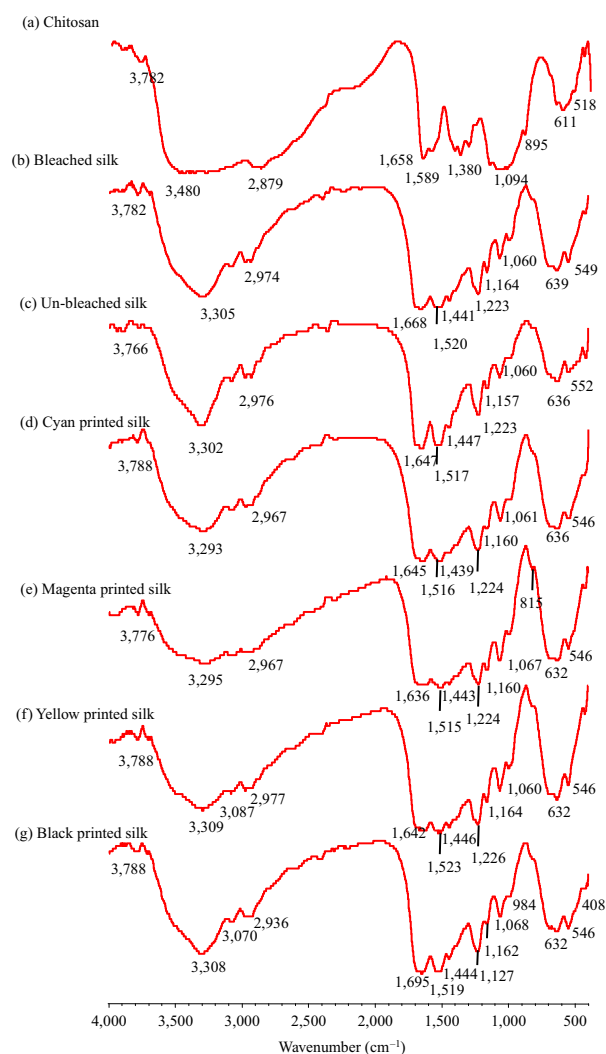
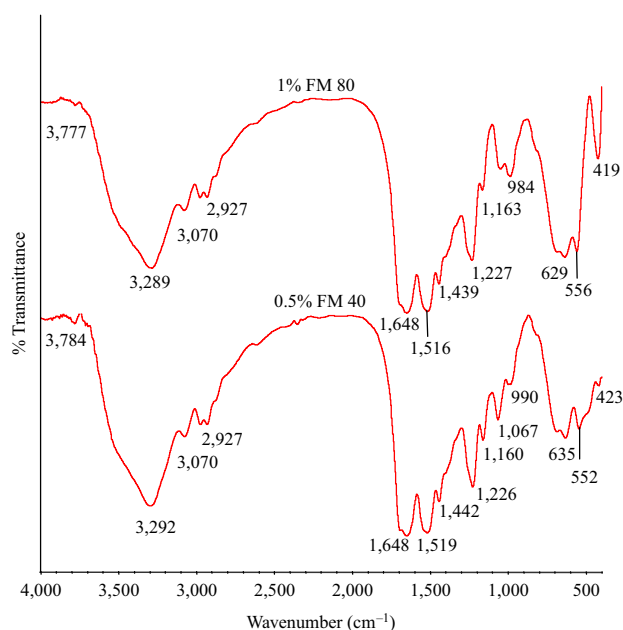
concentration (FM-80) increases the viscosity of the padding solution (Figure 4(b)). Spectrum (a) of Figure 5 shows IR peaks of the non-treated silk fabric. In spectrum (b) of Figure 5 for chitosan-treated silk, a sharp strong NH_3^+ stretching band in the $3,278\text{--}2,901\text{ cm}^{-1}$ region resulting from superimposed O—H and NH_3^+ stretching band are stronger than those in spectrum (a). The stronger peaks in spectrum (b) at $1,050\text{--}989\text{ cm}^{-1}$ are assigned for the symmetric C—O—C stretching of chitosan. The asymmetric N—H stretching at $1,617\text{--}1,510\text{ cm}^{-1}$ and more weakly near

Figure 5 FTIR-ATR spectra of (a) the non-treated silk and (b) chitosan-treated silk

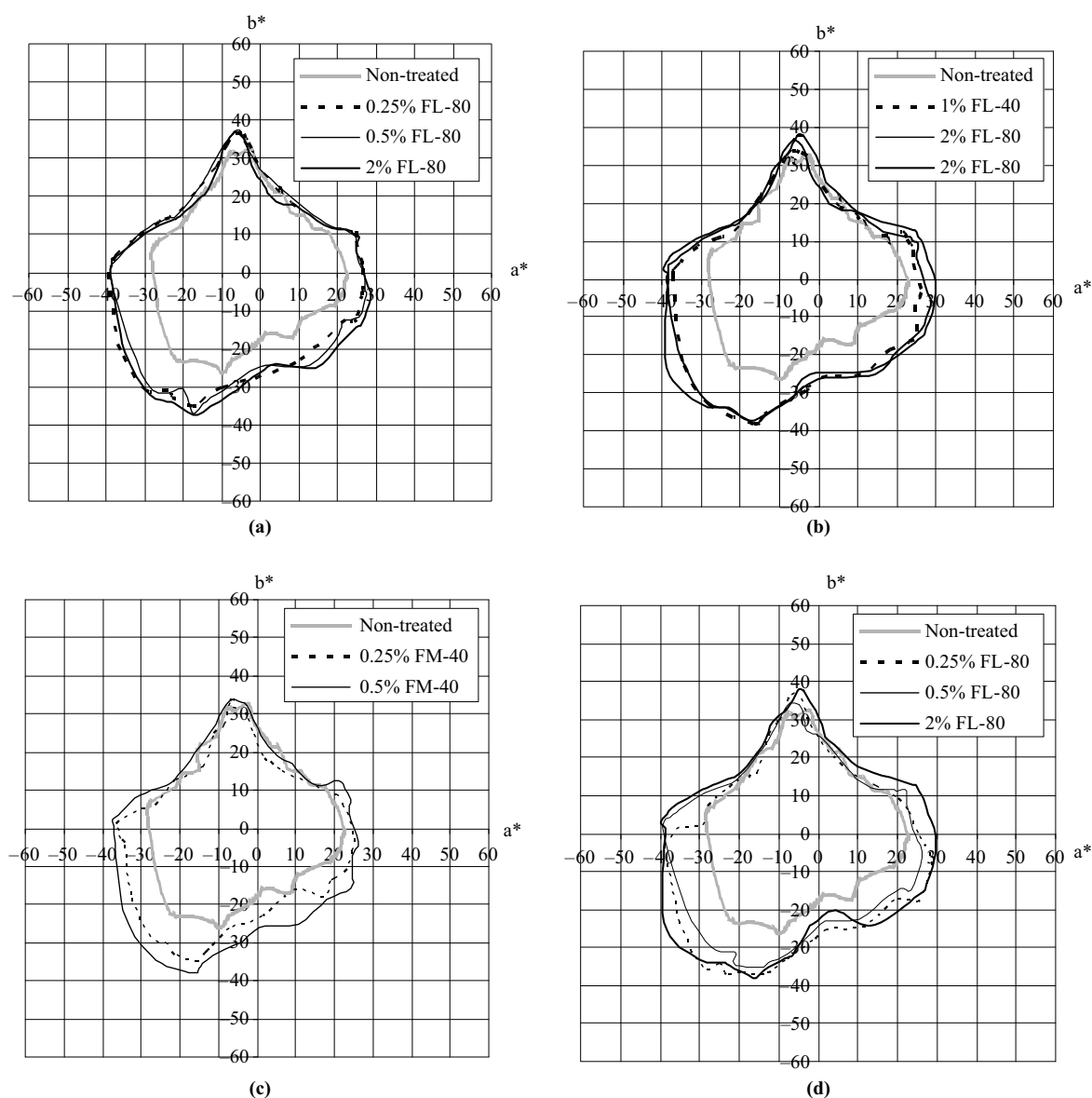


With the same degree of deacetylation (87–88 per cent), the three types of chitosan that were used in this work did not display any different chemical properties, for example with the IR spectra of silk fabric padded with the two types of chitosan, FM-80 and FM-40 (Figure 6). Moreover, the ink jet ink-printed silk fabrics that had previously been padded with chitosan solutions gave similar chemical surface properties, as shown by their IR spectra (Figure 7). Hydrogen peroxide bleaching of the fabric is to remove surface sericin and other waxy materials resulting in a clean and white surface for better wetting, adhesion and image permanency. Interactions between the ink and the substrate cannot be easily detected by simple spectroscopic methods because of the substrate dominance in comparison with the chitosan-treating solution. Thus, the dual objectives of developing improvements in the printing process with improvements in the colour-related properties were achieved initially. Further investigation of the extent of colour gamut expansion could provide an indication of the synergistic effects provided by the inks and the chitosan pre-treatment solution.

For the three different types of chitosan, and across all the tested ranges of chitosan concentrations used, the colour gamuts (a^*-b^* , L^*-a^* , L^*-b^* and $L^*a^*b^*$) of the pre-treated fabrics were overall greater, i.e. a larger gamut volume was attained than those printed colours applied to the non-treated



Increasing the pre-treatment solution concentration for each of the three different molecular weight chitosans, resulted in a wider colour space where the chroma of the cyan colours, at many hue angles, became significantly saturated. A similar trend was also observed with the magenta colours but to a smaller extent. The colour strength of the fabrics printed following with the chitosan pre-treatments increased consistently, the chitosan concentration in the pre-treatment was increased. Amongst the three molecular weight

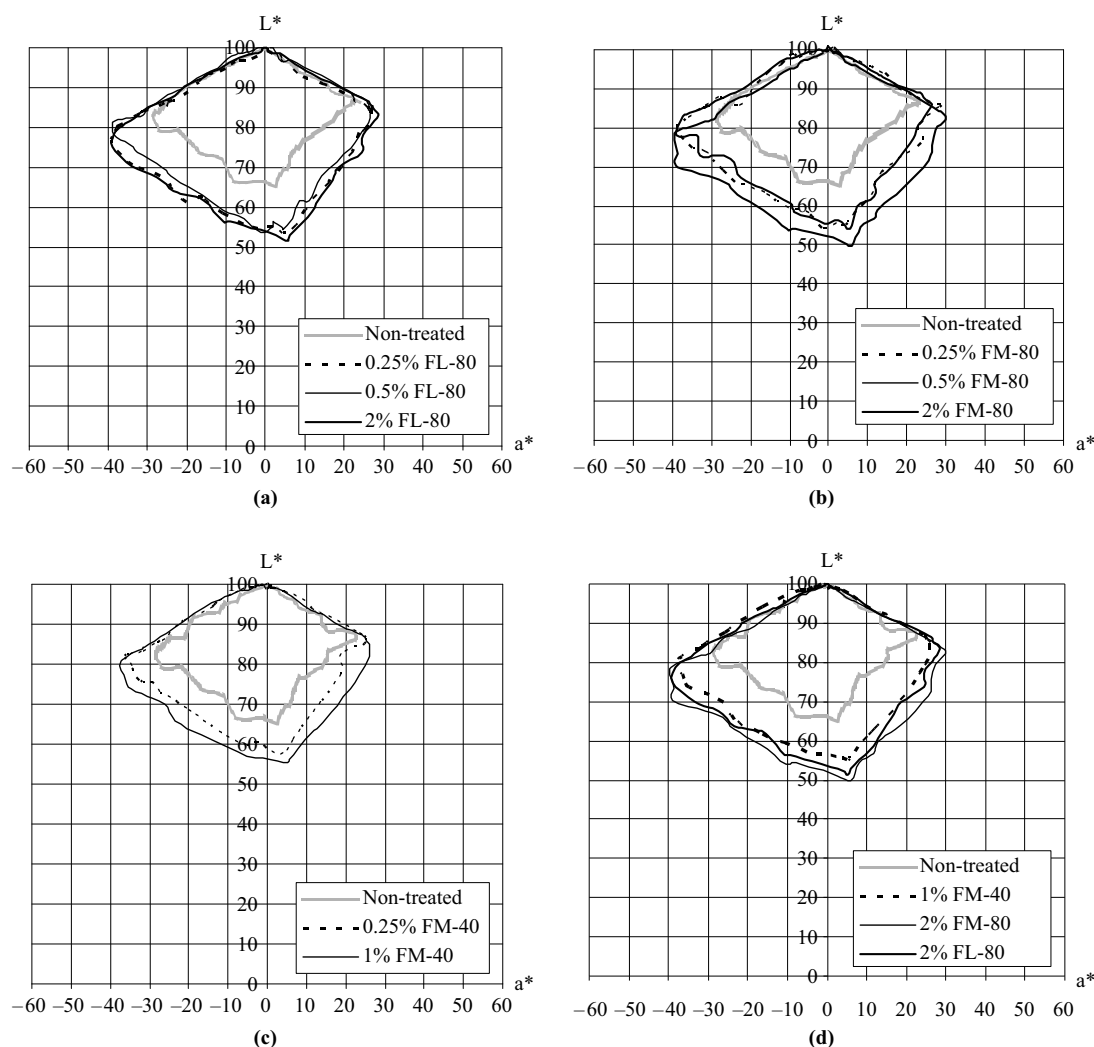
Figure 8 a^* - b^* diagrams of the non-treated silk and the silk pre-treated with chitosan of different molecular weights and loadings

chitosans tested, the pre-treatment solution containing the medium molecular weight chitosan (FM-80), prepared at the highest concentration (2 per cent w/v), gave the largest colour gamut (the combined $L^*a^*b^*$ in Figure 11). This finding correlates well with its highest solution viscosity (Figure 4). Chitosan pre-treatment improves the colour gamut of the printing inks because the chitosan molecule contains amino groups ($-NH_2$) that are protonated ($-NH_3^+$) under acidic conditions and can electrostatically interact with the sulphonate or carboxylate groups on the pigment surfaces, even at a low chitosan concentration (Jocic *et al.*, 2005). Thus, the pre-treatment solutions provide the silk fabric with additional cationic functional groups that enhance the absorption of more ink on to the fabric surface. A colour gamut expansion on the treated fabric surfaces is related to the increases in the chroma (Figure 8). The function of the

pre-treatment is to block ink penetration and to give retention of ink on the pre-treated surface. One of the chief concerns with treating silk fabric with chitosan is the increased stiffness of the treated fabrics (Leelajariyakul *et al.*, 2008; Phattananurudee *et al.*, 2009), and so it is desirable to use the smallest amount of chitosan in the pretreatment solution as possible to fulfil the need for a specific purpose.

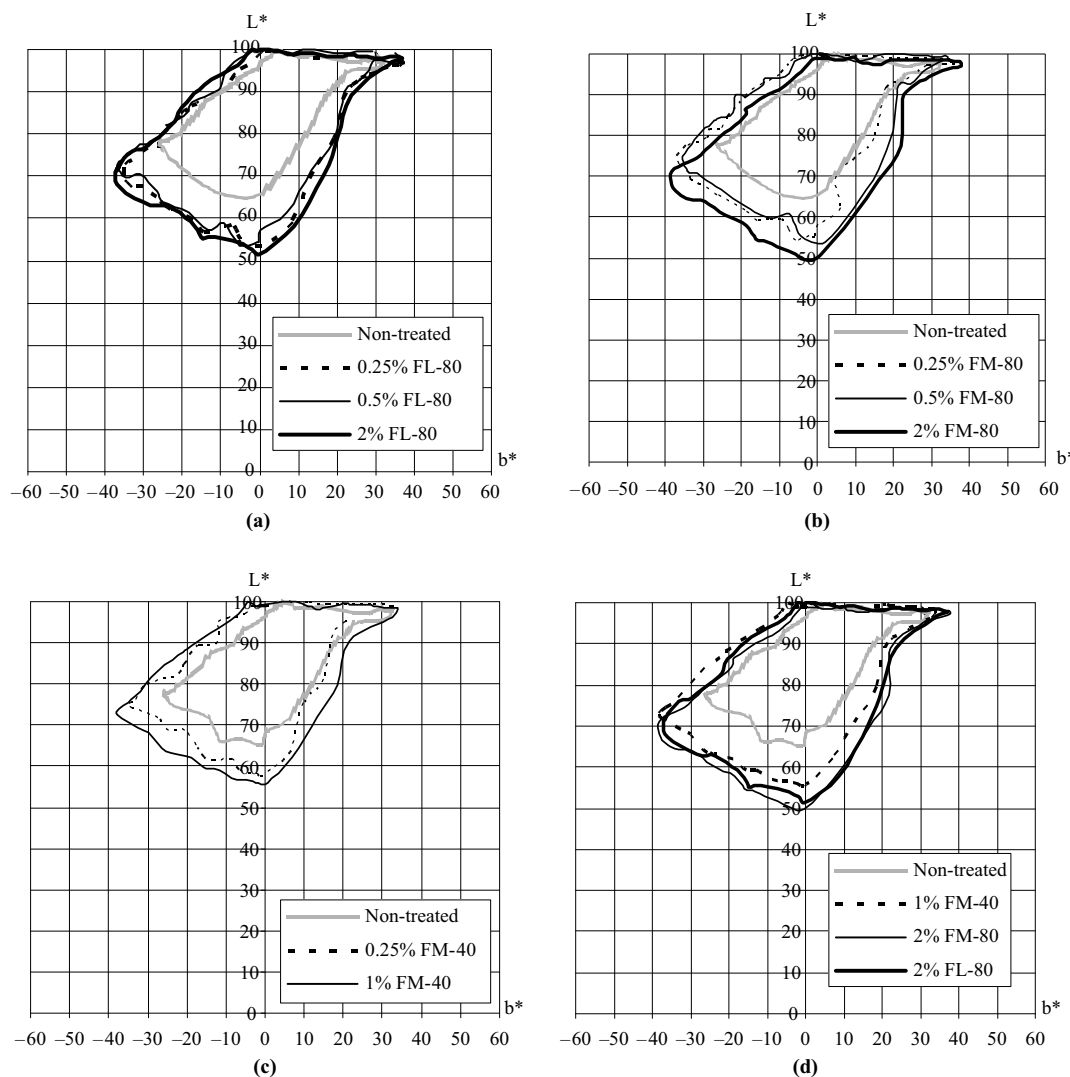
Wash fastness

The inks contained the S-711 acrylic binder with its T_g of -31°C . This binder is designed to form a flexible film on the silk surface, giving the required properties to the fabric. The effect of the chitosan pre-treatment of silk on their wash fastness following printing was determined by following the K/S of the coloured fabrics, measured before and after the washings. The K/S values of the treated fabrics before and

Figure 9 L^* - a^* diagrams of the non-treated silk and the silk pre-treated with chitosan of different molecular weights and loadings

after washing were in unchanged range (0.4–0.5) for cyan and magenta inks, whereas the ranges of 1–1.3 and 1.7–2.5 were found in the yellow and black inks, respectively, depending on the treatment levels. The wash fastness of the printed fabrics was thus excellent for all of the pre-treatments (Table III), and the relative colour strength of the printed silk fabrics after washing is summarized in Table IV where a very small change in the K/S before and after washing, across all of the pre-treatments, was observed. Also, the pre-treatments moderately improved the wash fastness. The relative colour strength of the non-treated fabric was 0.82 for all colours, and the treated ones in the range of 0.9–1.2 for the cyan, magenta, yellow, and black inks, depending on the chitosan molecular weights and solution concentration. The chitosan pre-treatment of silk fabric thus enhanced the wash fastness properties. Comparing between the different chitosans used in the pre-treatments, the chitosan molecular weight and concentration had a slight effect on the wash fastness and colour gamut. At 2 per cent w/v of FL-80 and 1 per cent w/v of FM-80 and FM-40, all of the K/S values increased, especially for the black ink, when compared with the results on the coloured non-treated fabrics.

The amino group in chitosan has a pKa value of about 6.5. Thus, chitosan is positively charged and soluble in acidic to neutral solutions with a charge density that is dependent on the pH and on the percentage of deacetylation that the chitosan contains. The amino groups are protonated to (NH_3^+) (a nucleophilic group) under slightly acidic pH conditions allowing ionic (electrostatic) interactions with electrophilic centres, such as carboxylate ($-\text{COO}^-$) or sulphonate ($-\text{SO}_3^-$) groups. The proposed electronic interaction is shown in Figure 12. A large pigment molecule with a correspondingly large surface area will provide a large number of potential reactive sites, such as the N–H, O–H or C=O groups. Thus, hydrogen-bonding interactions can occur to increase the strength of the binding over that of only the electrostatic interactions (Crini and Badot, 2008). All of these interactions, whether electrostatic, polar or H bonding, between the colorant and silk fabric, produce printed silks with an excellent wash fastness (Provost *et al.*, 2009). Here, the cationic amino moiety increases the absorption potential of these negatively charged inks via an ionic bonding mechanism (Noguchi and Shirota, 2006; Li and Sun, 2007), since the amino groups in the pre-treating agent and the amino groups in

Figure 10 L^* - b^* diagrams of the non-treated silk and the silk pre-treated with chitosan of different molecular weights and loadings

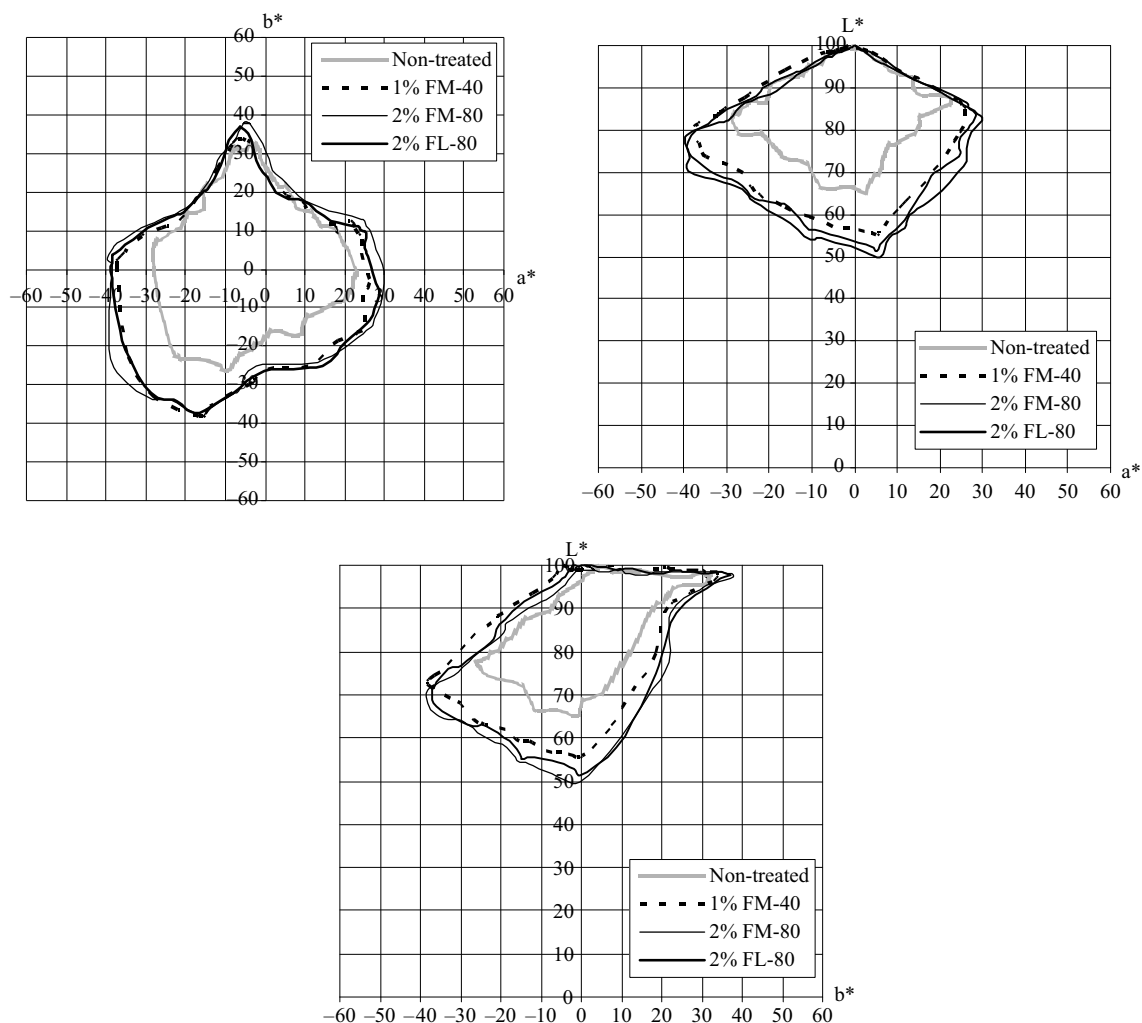
the silk are both protonated and can interact with the anionically surface-modified pigment particles, resulting in an increased interaction, increased ink binding and, consequently, an increase in the K/S values.

Carbon black pigments can contain hydrophilic carboxylic groups and hydroxyl groups from the production process (spectrum (d) in Figure 1). Such pigments can be additionally surface modified by the use of diazonium salts that contain the sulphonate functional group (Belmont, 1994). Thus, the carbon black pigments can be better dispersed under acidic conditions. It is known that carbon black is one of the most difficult-to-disperse pigments. Therefore, additional effective functional groups on the surface provide opportunity for the better development of superior black inks. The calculated K/S values, coupled with visual assessment, support the contention that these inks easily meet the appropriate performance criteria in that the visual ink density and the associated adhesion are both acceptable. The solid content of 50.7 per cent w/w of the Vanatex S-711 in the inks plays a very important role in the ability of the ink to interact with the padded chitosan on the silk fabric, i.e. holding more ink on

the surface. This would be expected to increase the colour gamut and the wash fastness.

Crock fastness

From the data given in Table V, it is clear that the non- and pre-treated printed silk fabrics provide the required minimal level of a good dry/wet crock fastness, at levels of 4–5. This is because the inks contained the binder (S-711 acrylic polymer) whose film is flexible (T_g of -31°C) at room temperature and can withstand the rubbing processing. The chitosan pre-treatment slightly improved the fabric dry/wet crock fastness (at the 4/5 level). In dry conditions, chitosan loadings from pre-treatment with 0.25 and 0.5 per cent w/v solutions did not affect the dry rub resistance. However, a decline in the rub resistance (at the 4 level) was found under wet conditions, presumably due to a water-acid effect. For all of the chitosan pre-treatments, the dry crock fastness results were better than those of the wet crock fastness. The wet crock fastness was slightly decreased when the chitosan loading solution was more than 0.5 per cent w/v, but was essentially the same for the three different molar mass chitosans evaluated here (Leelajariyakul *et al.*, 2008). The crock and the wash fastness

Figure 11 $L^*a^*b^*$ of the untreated silk and the treated silk pre-treated with chitosan of different molecular weights and loadings**Table III** K/S of the printed silk fabrics before and after washing

Condition of fabric treatment	K/S of the printed silk with the inks							
	Cyan		Magenta		Yellow		Black	
	Before washing	After washing	Before washing	After washing	Before washing	After washing	Before washing	After washing
Non-treated								
Chitosan ^a (FL-80)	0.25	0.42 ± 0.00	0.38 ± 0.01	0.45 ± 0.03	0.41 ± 0.02	1.18 ± 0.05	0.96 ± 0.07	1.41 ± 0.01
	2	0.38 ± 0.04	0.40 ± 0.06	0.39 ± 0.02	0.40 ± 0.02	1.10 ± 0.04	1.08 ± 0.08	1.73 ± 0.03
Chitosan ^b (FM-80)	0.25	0.48 ± 0.04	0.47 ± 0.02	0.45 ± 0.03	0.45 ± 0.02	1.30 ± 0.01	1.28 ± 0.02	2.18 ± 0.04
	1	0.4 ± 0.02	0.41 ± 0.02	0.37 ± 0.01	0.38 ± 0.01	0.99 ± 0.04	0.96 ± 0.03	2.54 ± 0.19
	2	0.45 ± 0.01	0.47 ± 0.03	0.47 ± 0.02	0.48 ± 0.01	1.29 ± 0.05	1.27 ± 0.06	2.20 ± 0.07
Chitosan ^c (FM-40)	0.25	0.56 ± 0.06	0.55 ± 0.06	0.53 ± 0.01	0.51 ± 0.01	1.26 ± 0.09	1.23 ± 0.09	2.19 ± 0.14
	1	0.42 ± 0.02	0.44 ± 0.02	0.41 ± 0.01	0.44 ± 0.02	1.14 ± 0.01	1.14 ± 0.04	1.92 ± 0.03
	2	0.45 ± 0.03	0.43 ± 0.02	0.45 ± 0.03	0.42 ± 0.03	1.34 ± 0.04	1.28 ± 0.05	2.54 ± 0.19

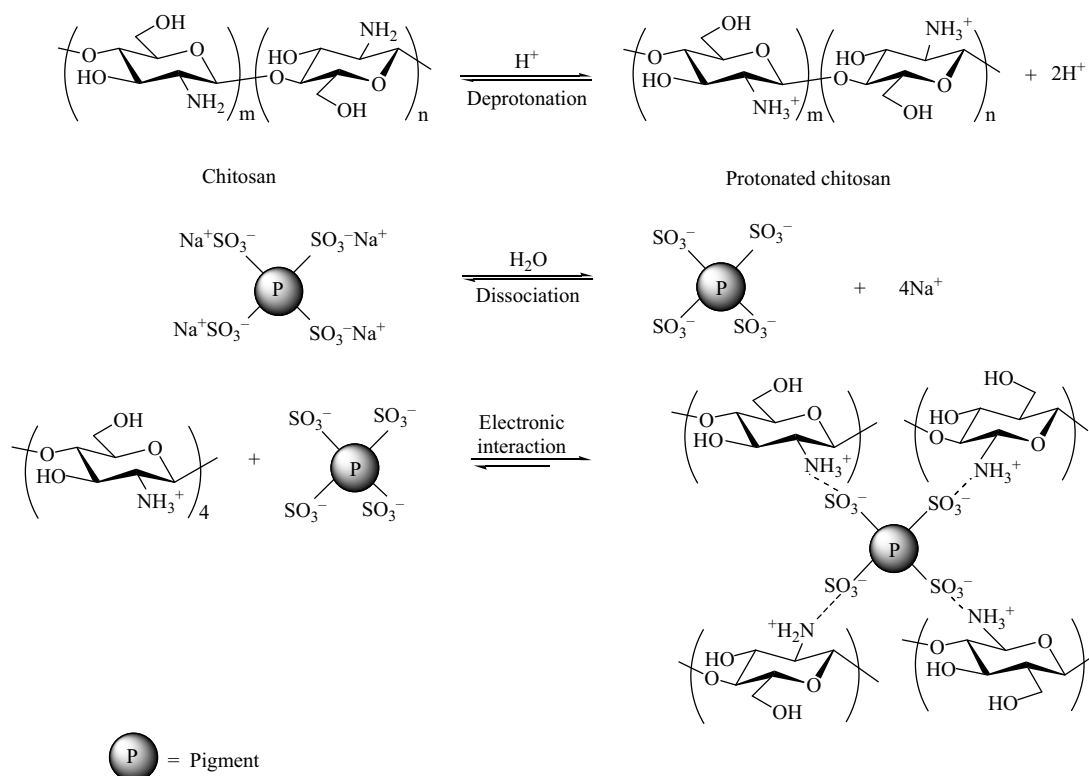
Notes: ^aMW = 1.2×10^5 g mol⁻¹; ^bMW = 3.7×10^5 g mol⁻¹; ^cMW = 8.5×10^5 g mol⁻¹; all have an 87–88% degree of *N*-deacetylation; data are shown as the mean ± 1 (SD) and are derived from three replicates

Table IV Relative colour strength of the printed silk fabrics after washing

Condition of treatment		Average of relative colour strength			
		Cyan	Magenta	Yellow	Black
Non-treated		0.82 ± 0.01	0.90 ± 0.00	0.81 ± 0.03	0.82 ± 0.02
Chitosan ^a (FL-80) (%(w/v))	0.25	1.04 ± 0.04	1.04 ± 0.01	0.98 ± 0.03	1.22 ± 0.00
	2	0.99 ± 0.03	1.00 ± 0.02	0.98 ± 0.02	1.23 ± 0.00
Chitosan ^b (FM-80) (%(w/v))	0.25	1.03 ± 0.03	1.03 ± 0.00	0.98 ± 0.01	0.98 ± 0.07
	1	1.03 ± 0.03	1.03 ± 0.01	1.04 ± 0.01	1.03 ± 0.02
	2	0.98 ± 0.00	0.96 ± 0.00	0.98 ± 0.01	0.98 ± 0.02
Chitosan ^c (FM-40) (%(w/v))	0.25	1.06 ± 0.01	1.06 ± 0.01	1.00 ± 0.02	1.01 ± 0.01
	1	0.95 ± 0.00	0.93 ± 0.00	0.96 ± 0.01	0.95 ± 0.03

Notes: ^aMW = 1.2×10^5 g mol⁻¹; ^bMW = 3.7×10^5 g mol⁻¹; ^cMW = 8.5×10^5 g mol⁻¹; all have an 87–88% degree of *N*-deacetylation; data are shown as the mean ± 1 (SD) and are derived from three replicates

Figure 12 Proposed scheme for the chitosan pre-treating agent and anionic pigment interactions



are both governed by the strength of adhesion and by the interfacial bonding between the pigment particles/ink binder/pre-treated fabric surfaces.

Conclusion

Anionically surface-modified organic pigment dispersions were used to prepare four coloured polyacrylate binder-based ink jet inks within the pH range of 8–9. These inks exhibited Newtonian flow in which the viscosity at 25°C remained constant at 2.5 mPa s under a 330 s⁻¹ shear-strain rate. The surface tension of the inks was approximately 44 mN m⁻¹ at 25°C, and the average particle sizes were approximately 100 nm. The zeta-potential of the inks was

strongly negative, in the range of –42 to –51 mV, and they were stable for over ten months (for the magenta and black inks) or one year (for the cyan and yellow inks), as confirmed by SEM and TEM micrographs. The inks were shown to be suitable for printing by the piezo ink jet method on both non-treated and chitosan pre-treated silk fabrics. The anionically surface-modified pigmented ink interacted with the protonated amino groups of chitosan giving improved ink deposition and fixation on the fabric surface. The colour gamuts of the treated fabrics were wider with the chroma of the cyan colour being the most improved followed by that of the magenta colour. The chitosan pre-treatment produced the better crock fastness and wash fastness of the printed fabrics than the non-treated ones.

Table V Crock fastness of the non-treated and chitosan pre-treated silk fabrics

Condition of treatment	Crock fastness							
	Dry stage				Wet stage			
	C	M	Y	K	C	M	Y	K
Non-treated	4	4	4/5	4	4	4	4	4
Chitosan (FL-80) (%(w/v))	0.25	4/5	4/5	4	4/5	4/5	4/5	4
	0.5	4/5	4/5	4	4/5	4/5	4/5	4
	1	4	4	4	4/5	4	4	3/4
	2	4	4	4	4/5	4	4	3/4
Chitosan (FM-80) (%(w/v))	0.25	4/5	4	5	4/5	4/5	4/5	4
	0.5	4/5	4/5	4	4/5	4/5	4/5	4
	1	4/5	4/5	4	4/5	4/5	4	3/4
	2	4/5	4/5	4	4/5	4	4	3/4
Chitosan (FM-40) (%(w/v))	0.25	4/5	4/5	4	4/5	4/5	4/5	4
	0.5	4/5	4/5	4	4/5	4/5	4/5	4
	1	4/5	4/5	4	4/5	4/5	4/5	4

Note: 1, 2, 3, 4 and 5 are the rating levels for poor, fair, fairly good, good and excellent, respectively

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The Journal of the Royal Institute of Thailand

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การวัด Bidirectional Reflectance Distribution Function และการจำลอง แบบการสะท้อนแสงของผ้าไหม*

สุดา เกียรติคำจรวงศ์**

ภาควิชาชีววิทยา สำนักวิทยาศาสตร์

ราชภัฏบรียรัมย์

บุญเกียรติ นายนพประสิทธิ์***

ชวาล กุรุพิพัฒน์***

บทคัดย่อ

ผ้าไหมเป็นสิ่งทอที่มีความมันวาวมากกว่าสิ่งทอประเภทอื่น เนื่องจากผ้าไหมมีลักษณะการสะท้อนแสงที่มีความเฉพาะตัว ดังนั้น งานวิจัยนี้จึงศึกษาลักษณะการสะท้อนแสงของผ้าไหม โดยวัดค่าบิอาร์ดีเอฟ (BRDF, Bidirectional Reflectance Distribution Function) และนำเสนอแบบจำลองการสะท้อนแสงของพื้นผิวผ้าไหมที่คำนึงถึงการสะท้อนของแสงพุ่งและแสงกล้ำ โดยใช้วิธีการหาค่าความผิดพลาดกำลังสองน้อยสุด (least-square error) หาความสัมพันธ์ระหว่างค่าที่วัดกับแบบจำลอง เมื่อทดสอบแบบจำลองที่ได้พบว่า แบบจำลองที่นำเสนอมีความสัมพันธ์กับบิอาร์ดีเอฟที่วัดได้ของผ้าไหมมากกว่าแบบจำลองการสะท้อนแสงที่มีอยู่ทั่วไป ได้แก่ แบบจำลองการสะท้อนแสงของฟอง แบบจำลองการสะท้อนแสงของโอเรนและนaylor และแบบจำลองการสะท้อนแสงของทอร์แรนซ์และสเปร์โรว์ ผู้วิจัยสามารถพยากรณ์ค่าการสะท้อนแสง วิเคราะห์ และอธิบายลักษณะการสะท้อนแสงของผ้าไหมได้จากแบบจำลองที่นำเสนอ

คำสำคัญ : บิอาร์ดีเอฟ, ผ้าไหม, แบบจำลองการสะท้อนแสง

คำนำ

ในยุคดิจิทัลที่การค้าขายในอินเทอร์เน็ตได้รับความนิยมอย่างกว้างขวาง ความสวยงามและเหมือนจริงของสินค้าที่เป็นผ้าไหม หรือผลิตภัณฑ์อื่นจากผ้าไหมเป็นสิ่งที่มีผลต่อการตัดสินใจซื้อของผู้บริโภค ความมันวาว (gloss) เป็นปัจจัยด้านคุณูปภาพที่สำคัญอย่างหนึ่งที่เกิดจากสมบัติการสะท้อนแสงของผ้าไหม และมุมของแสงที่ตกกระทบและมุมมอง พื้นผิววัตถุใด ๆ สามารถเกิดการสะท้อนแสงใน ๓ ลักษณะ คือ การสะท้อนแสงแบบพุ่ง (diffuse reflection) การสะท้อน

แสงแบบกล้ำ (specular reflection) และการสะท้อนแสงแบบผสมระหว่างแสงพุ่งกับแสงกล้ำ (mixed reflection) [Wyszecki and Stiles, 2000] ดังรูปที่ ๑



รูปที่ ๑ ลักษณะการสะท้อนแสงแบบพุ่ง แบบกล้ำ และแบบผสม

ปรกติพื้นผิววัตถุส่วนใหญ่มีการสะท้อนแสงแบบผสม วัตถุที่มีความมันวาวมากจะสะท้อนแสงกล้ำออกมา วัตถุที่มีผิวด้านหรือผิวไม่เรียบจะ

สะท้อนแสงพุ่งออกมามาก ลักษณะการสะท้อนแสงเหล่านี้ ทำให้มีผู้สร้างแบบจำลองการสะท้อนแสงของพื้นผิว

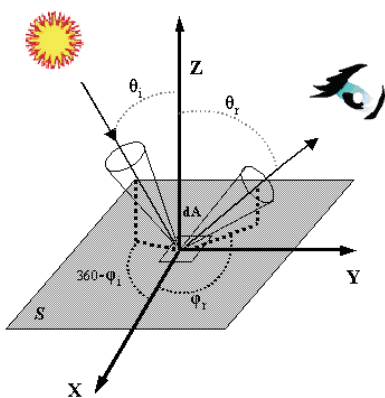
* บทความนี้ปรับปรุงจากการบรรยายผลงานทางวิชาการในที่ประชุมสำนักวิทยาศาสตร์ เมื่อวันที่ ๑๗ กุมภาพันธ์ พ.ศ. ๒๕๕๓

** ภาควิชาวิทยาศาสตร์ทางภาพถ่ายและเทคโนโลยีทางการพิมพ์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ ๑๐๓๓๐



วัตถุขึ้นมา เพื่อใช้พยากรณ์ค่าการสะท้อนแสงของพื้นผิววัตถุและได้นำไปใช้ในโปรแกรมสำหรับสร้างภาพสามมิติในคอมพิวเตอร์ เช่น ภาพแบบจำลองสินค้า ภาพการ์ตูนแอนิเมชัน ภาพยนตร์ ความเหมือนจริงของรูปที่สร้างขึ้นด้วยโปรแกรมเหล่านี้ขึ้นอยู่กับความสามารถในการจำลองค่าการสะท้อนแสงของแบบจำลอง แบบจำลองชนิดหนึ่งอาจเหมาะกับวัตถุที่มีลักษณะผิวอย่างหนึ่ง แต่อาจไม่เหมาะกับวัตถุที่มีพื้นผิวแตกต่างออกไป

Bidirectional Reflectance Distribution Function (BRDF) เป็นฟังก์ชันการสะท้อนแสงในทิศทางต่าง ๆ ของพื้นผิววัตถุ เมื่อเปลี่ยนมุมตกกระทบของแสงแบบจำลอง โดยทั่วไปกำหนดให้การสะท้อนแสงรวมเป็นผลรวมระหว่างการสะท้อนแสงแบบฟุ้งกับการสะท้อนแสงแบบกล้ำ ซึ่งมีตัวแปรเป็นมุมตกกระทบและมุมรับภาพ [Dana et al., 1999] ดังรูปที่ ๒



รูปที่ ๒ BRDF ของพื้นผิววัตถุใด ๆ

สมการรูปแบบทั่วไปของ BRDF สามารถเขียนได้ดังสมการที่ ๑

$$L_r = k_d L_d + k_s L_s \quad (๑)$$

โดยที่

L_r คือ การสะท้อนแสงของวัตถุ

L_d คือ การสะท้อนแสงแบบฟุ้ง (diffuse reflection)

L_s คือ การสะท้อนแสงแบบกล้ำ (specular reflection)

k_d คือ สัมประสิทธิ์ของการสะท้อนแสงแบบฟุ้ง (diffuse components reflection coefficient)

k_s คือ สัมประสิทธิ์ของการสะท้อนแสงแบบแสงกล้ำ (specular components reflection coefficient)

$$k_d + k_s = ๑$$

แบบจำลองการสะท้อนแสงของพื้นผิววัตถุมีอยู่ด้วยกันหลายแบบ ซึ่งสามารถพยากรณ์การสะท้อนแสงแบบฟุ้งและแบบกล้ำที่แตกต่างกัน แบบจำลองพื้นฐานที่มองพื้นผิววัตถุมีการสะท้อนแสงแบบอุดมคติคือแบบจำลองการสะท้อนแสงแบบแลมเบิร์ตเนียน (Lambertian reflection model) [Dana et al., 1999] ส่วนแบบจำลองการสะท้อนแสงของโอเรนและนayar (Oren and Nayar reflection model) [Oren and Nayar, 1995] สามารถพยากรณ์การสะท้อนแสงแบบฟุ้งได้แม่นยำมาก เพราะรวมลักษณะความขรุขระของพื้นผิววัสดุไว้ในแบบจำลอง

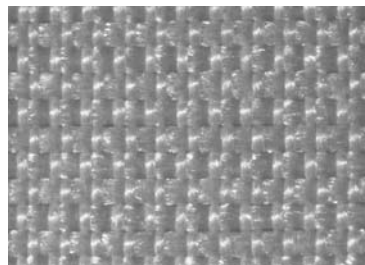
ด้วย แบบจำลองที่พยากรณ์การสะท้อนแสงแบบกล้ำ ได้แก่ แบบจำลองของทอร์เรนซ์และสแปร์โรว์ [Torrance and Sparrow], แบบจำลองการสะท้อนแสงของคุก-ทอร์เรนซ์ (Cook-Torrance reflection model) [Dana et al., 1999] และแบบจำลองการสะท้อนแสงของบลินน์ (Blinn reflection model) [Blinn, 1977] แบบจำลองแต่ละแบบได้คำนึงถึงปริมาณแสงสะท้อนแบบกล้ำที่รวมปัจจัยด้านความขรุขระของพื้นผิวเอาไว้ด้วย และแบบจำลองที่เหมาะสมสำหรับพยากรณ์การสะท้อนแสงแบบผสม ก็คือ แบบจำลองการสะท้อนแสงของฟอง (Phong reflection model) [Phong, 1975] ซึ่งเหมาะสมกับพื้นผิวเรียบ ๆ เช่น พลาสติก ในขณะที่แบบจำลองการสะท้อนแสงของวาร์ด (Ward reflection model) [Ward, 1992] และแอชกิน-เชอร์ลีย์ (Ashikmin-Shirley reflection model) [Ashikmin and Shirley, 2000] ได้ปรับปรุงแบบจำลองการสะท้อนแสงของฟอง (Phong) ให้เหมาะสมมากขึ้นกับลักษณะพื้นผิวที่หลากหลายและไม่สามารถจำลองแบบใดที่สร้างขึ้นเพื่อใช้กับการสะท้อนแสงของพื้นผิวผ้าไหม โดยเฉพาะ ดังนั้น งานวิจัยนี้จึงศึกษาลักษณะการสะท้อนแสงของพื้นผิวผ้าไหม โดยการวัดค่าบีอาร์ดีเอฟและนำค่าที่วัดได้มาสร้างเป็นแบบจำลองการสะท้อนแสงที่สัมพันธ์กับค่าการสะท้อนแสงของพื้นผิวผ้าไหม



อุปกรณ์และวิธีการ

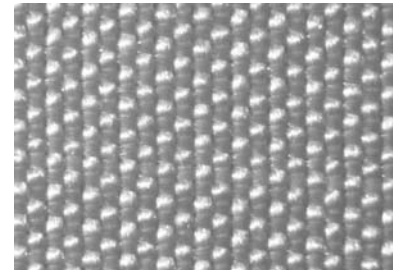
การทดลองนี้ได้ใช้ผ้าไหมสีขาวที่ทอด้วยลายขัด ๓ ตัวอย่าง ผ้าไหมตัวอย่างที่ ๑ ใช้เส้นไหม ๒ เส้นในการทอและเส้นไหมมาจากบ้านไหมไทยคอลเล็กชัน ผ้าไหมตัวอย่างที่ ๒ และตัวอย่างที่ ๓ ใช้เส้นไหม ๒ และ ๔ เส้นในการทอและเส้นไหมมาจากบ้านจิม ทอปั้มสัน ซึ่งมีลักษณะพื้นผิวตามรูปที่ ๓ ถึง รูปที่ ๕ ตามลำดับ งานวิจัยนี้ได้แบ่งการทดลองออกเป็น ๒ ขั้นตอน ได้แก่ การทดลองตอนที่ ๑ เป็นการวัดค่าบีอาร์ดีเอฟของผ้าไหมทั้ง ๓ ตัวอย่าง ด้วยเครื่องโกนิโอสเปกโตรโฟโตมิเตอร์ (ยี่ห้อ มูราคามิ รุ่น จีเอสพี-๒๕ ประเทศญี่ปุ่น) ซึ่งได้วัดค่าที่ศูนย์การวิจัยฟรอนเทียร์เมดิคัลเอนจิเนียริง (Research Center for Frontier Medical Engineering) แห่งมหาวิทยาลัยชิบะ ประเทศญี่ปุ่น โดยใช้มุมของแหล่งกำเนิดแสง ที่ ๐, -๑๕, -๓๐, -๔๕, -๖๐ และ -๗๕ องศา และใช้มุมรับภาพที่ -๗๕, -๖๐, -๔๕, -๓๐, -๑๕, ๐, ๑๕, ๓๐, ๔๕, ๖๐ และ ๗๕ องศา ตามลำดับ แล้วจึงนำค่าบีอาร์ดีเอฟที่ วัดได้มาเขียนกราฟเชิงขั้ว เพื่อทำให้สามารถวิเคราะห์ลักษณะการสะท้อนแสงของพื้นผิวผ้าไหม พร้อมทั้งสามารถหาลักษณะเฉพาะตัวของการสะท้อนแสงของผ้าไหมได้ ผู้วิจัยได้ทดลองตอนที่ ๒ ซึ่งเป็นการนำเสนอแบบจำลองการสะท้อนแสงของพื้นผิวผ้าไหม โดยได้ปรับปรุงจากแบบจำลองการสะท้อนแสงของฟอง และทดสอบแบบจำลอง

ด้วยการหาความผิดพลาดที่น้อยที่สุดจากการเปรียบเทียบค่าที่คำนวณได้จากแบบจำลองการสะท้อนแสงที่นำเสนอ กับค่าบีอาร์ดีเอฟที่วัดได้จากผ้าไหมและความผิดพลาดจากการเปรียบเทียบค่าที่คำนวณได้จากแบบจำลองการสะท้อนแสงที่ใช้อยู่โดยทั่วไป ได้แก่แบบจำลองการสะท้อนแสงของฟองแบบจำลองการสะท้อนแสงของโอเรนและนาร์ยร์ แบบจำลองการสะท้อนแสงของวาร์ด และแบบจำลองการสะท้อนแสงของแอชกินินและเซอร์ลีย์ เริ่มแรกผู้วิจัยได้หาค่าคงตัวในแบบจำลองต่าง ๆ ที่สัมพันธ์กับลักษณะการสะท้อนแสงของผ้าไหม โดยใช้วิธีหาค่าความผิดพลาดกำลังสองน้อยสุด (least-square error) จึงคำนวณค่าการสะท้อนแสงจากแบบจำลองที่มีค่าคงตัวครบ แล้วจึงนำค่าที่คำนวณได้มาเปรียบเทียบหาความผิดพลาดกับค่าการสะท้อนแสงจากผ้าไหม โดยใช้วิธีการหาค่ารากที่สองของกำลังสองเฉลี่ย (root-mean-square error) เพื่อหาแบบจำลองการสะท้อนแสงที่สัมพันธ์กับค่าการสะท้อนแสงของผ้าไหมมาก

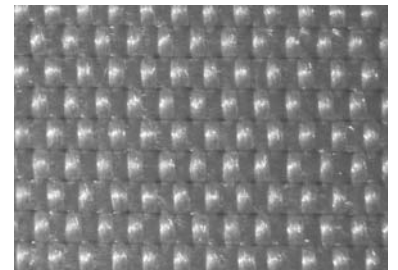


รูปที่ ๑
ลักษณะพื้นผิวของผ้าไหม
ตัวอย่างที่ ๑

ที่สุด



รูปที่ ๔
ลักษณะพื้นผิวของผ้าไหม
ตัวอย่างที่ ๒



รูปที่ ๕
ลักษณะพื้นผิวของผ้าไหม
ตัวอย่างที่ ๓

ผลการทดลองและวิจารณ์

ตอนที่ ๑ การวัดค่าบีอาร์ดีเอฟของผ้าไหม

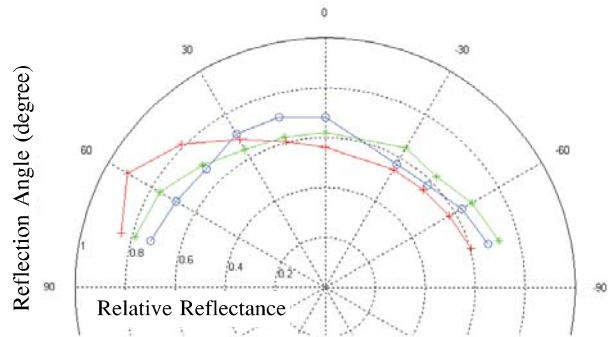
ค่าบีอาร์ดีเอฟของผ้าไหมสีขาว ๓ ตัวอย่าง สามารถเขียนเส้นโค้งในระบบพิกัดเชิงขั้ว ได้ตามรูปที่ ๖-๑๑ โดยใช้มุมของแหล่งกำเนิดแสง ๐, -๑๕, -๓๐, -๔๕, -๖๐ และ -๗๕ องศา ตามลำดับ เส้นโค้งทั้ง ๖ เส้นนี้ เส้นสีแดง (+) คือ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑ เส้นสีเขียว (*) คือ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๒ และเส้นสีน้ำเงิน (o) คือ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๓



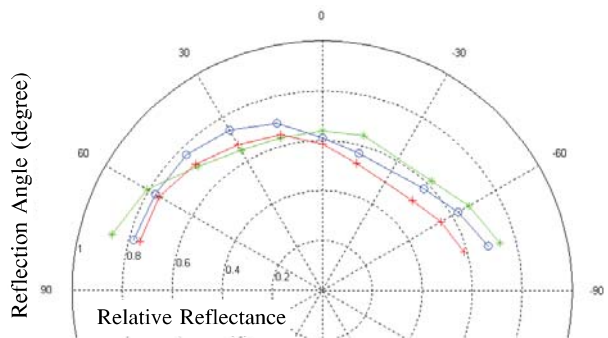
เส้นโค้งของค่าบีอาร์ดีเอฟของผ้าไหมแสดงให้เห็นลักษณะการสะท้อนแสงของพื้นผิวผ้าไหมซึ่งแสงกระจายตัวออกรอบทิศทาง และเป็นสมบัติของการสะท้อนแสงแบบฟุ้ง การกระจายตัวของค่าการสะท้อนแสงยังมีแนวโน้มที่เบ้ในทิศทางเดียวกันกับทิศทางของแหล่งกำเนิดแสง แสดงให้เห็นว่า ค่าการสะท้อนแสงกระจายตัวไปหนาแน่นในทิศทางตรงข้ามกับทิศทางของแหล่งกำเนิดแสง ซึ่งเป็นทิศทางของแสงสะท้อนแบบกล้ำ ดังนั้น จึงสรุปได้ว่าแสงที่สะท้อนออกจากพื้นผิวของผ้าไหมมีการสะท้อนแสงแบบผสม

อย่างไรก็ตาม เมื่อพิจารณาเส้นโค้งบีอาร์ดีเอฟของพื้นผิวผ้าไหมในส่วนที่เป็นแสงฟุ้ง พบว่าเป็นการสะท้อนแบบฟุ้งที่ไม่เป็นอุดมคติ เพราะแสงสะท้อนมีความเข้มไม่เท่ากันในแต่ละทิศทาง และเมื่อพิจารณาในส่วนที่เป็นแสงกล้ำจะพบว่า มุมของแสงสะท้อนที่มีความเข้มสูงสุดไม่เป็นมุมตรงข้ามกับมุมของแหล่งกำเนิดแสง แต่เกิดการเลื่อนตัวไปในทิศทางอื่น ซึ่งแสดงให้เห็นว่า แสงที่

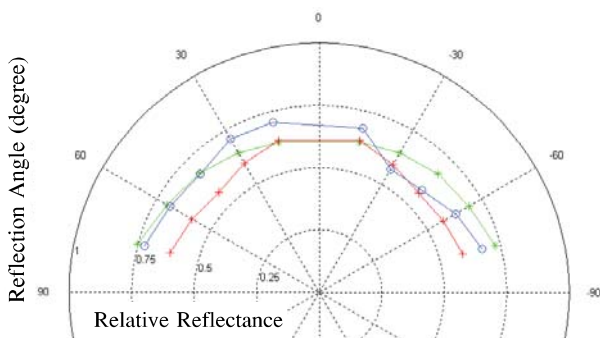
สะท้อนจากพื้นผิวผ้าไหมเกิด off-specular



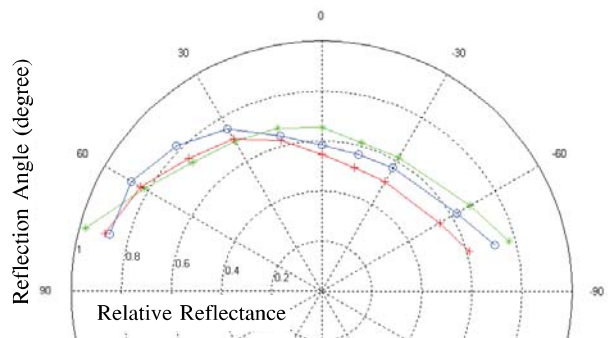
รูปที่ ๗ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑(+), ตัวอย่างที่ ๒(*) และตัวอย่างที่ ๓(๐) เมื่อใช้มุมตกกระทบ -๑๕ องศา



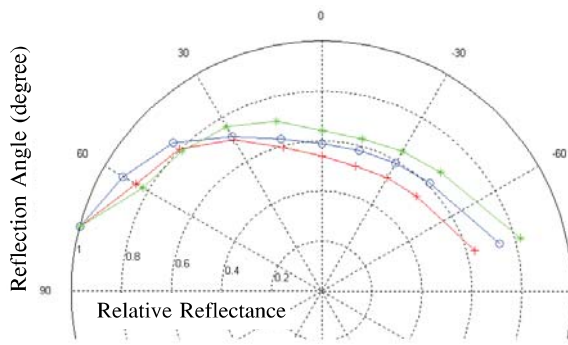
รูปที่ ๘ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑(+), ตัวอย่างที่ ๒(*) และตัวอย่างที่ ๓(๐) เมื่อใช้มุมตกกระทบ -๓๐ องศา



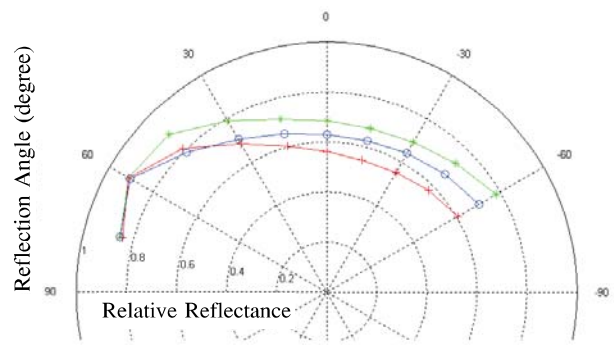
รูปที่ ๙ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑(+), ตัวอย่างที่ ๒(*) และตัวอย่างที่ ๓(๐) เมื่อใช้มุมตกกระทบ ๐ องศา



รูปที่ ๑๐ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑(+), ตัวอย่างที่ ๒(*) และตัวอย่างที่ ๓(๐) เมื่อใช้มุมตกกระทบ -๑๕ องศา



รูปที่ ๑๐ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑(+)
ตัวอย่างที่ ๒(*) และตัวอย่างที่ ๓(o) เมื่อใช้มุมตก
กระทบ -๖๐ องศา



รูปที่ ๑๑ ค่าบีอาร์ดีเอฟของผ้าไหมตัวอย่างที่ ๑(+)
ตัวอย่างที่ ๒(*) และตัวอย่างที่ ๓(o) เมื่อใช้มุมตก
กระทบ -๓๕ องศา

ตอนที่ ๒ การเสนอแบบจำลอง การสะท้อนแสงและการทดสอบ แบบจำลอง

เนื่องจากสมบัติการสะท้อนแสง
ของผ้าไหมเป็นการสะท้อนแสงแบบ
ผสม ผู้วิจัยจึงได้ปรับปรุงแบบจำลอง
การสะท้อนแสงของฟอง ในส่วนที่
เป็นการสะท้อนแสงแบบฟุ้งและแบบ
กล้ำ โดยเพิ่มพารามิเตอร์ α และ β
เพื่อปรับความกว้างของกรวยแสง และ
เพิ่มพารามิเตอร์ θ_{off} และ ϕ_{off} สำหรับ
ปรับมุมสะท้อนแสงแบบ off-specular
ดังแสดงในสมการที่ ๒

α คือ ค่าควบคุมความกว้างของกรวย
แสงฟุ้ง (diffuse lobe)

β คือ ค่าควบคุมความกว้างของกรวย
แสงกล้ำ (specular lobe)

θ_r คือ มุมรับภาพในแนวมุมเงย
(elevation)

θ_i คือ มุมของแหล่งกำเนิดแสงในแนว
มุมเงย (elevation)

θ_{off} คือ มุม off-specular ในแนวมุมเงย
(elevation)

ϕ_r คือ มุมรับภาพในแนวมุมกวาด
(azimuth)

ϕ_i คือ มุมของแหล่งกำเนิดแสงในแนว

กล่าวมาข้างต้น มาเปรียบเทียบกับค่า
บีอาร์ดีเอฟที่วัดจากผ้าไหม แล้ว
คำนวณหาความผิดพลาดโดยใช้วิธีหา
รากที่สองของกำลังสองเฉลี่ย (RMS)
ซึ่งแสดงผลในตารางที่ ๑

เมื่อพิจารณาผลจากตารางที่ ๑ แล้ว
จะเห็นว่า แบบจำลองการสะท้อนแสง
ที่นำเสนอนี้ให้ความผิดพลาดน้อยกว่า
แบบจำลองอื่น เช่น ผ้าไหมตัวอย่างที่
๓ ใช้มุมของแหล่งกำเนิดแสง -๖๐
องศา มีค่า RMS ของแบบจำลองที่
เสนออยู่ที่ ๐.๐๒๑๔ แบบจำลองของ
ฟองมีค่าอยู่ที่ ๐.๐๕๑๘ แบบจำลองของ

$$L(\theta_i, \theta_r, \phi_i, \phi_r) = k_d \cos^\alpha(\theta_r, \phi_r) + k_s \cos^\beta[\theta_r + (\theta_i - \theta_{off}), \phi_r + (\phi_i - \phi_{off})] \quad (๒)$$

โดยที่

L คือ ค่าการสะท้อนแสงของผ้าไหม
 k_d คือ สัมประสิทธิ์ความเข้มแสงของ
แสงฟุ้ง
 k_s คือ สัมประสิทธิ์ความเข้มแสงของ
แสงกล้ำ

มุมกวาด (azimuth)
 ϕ_{off} คือ มุม off-specular ในแนวมุม
กวาด (azimuth)

ผู้วิจัยได้ทดสอบแบบจำลอง
โดยนำค่าบีอาร์ดีเอฟที่คำนวณจาก
แบบจำลองการสะท้อนแสงต่าง ๆ ที่

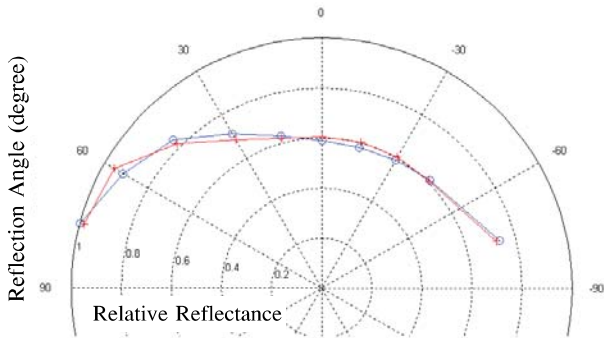
โอเรนและน่ายาร์อยู่ที่ ๐.๑๖๑๐
แบบจำลองของวาร์ด อยู่ที่ ๐.๑๐๓๖
และแบบจำลองของแอซิกมิน อยู่ที่
๐.๐๘๔๓ ซึ่งแสดงว่า ค่าบีอาร์ดีเอฟที่
ได้จากแบบจำลองการสะท้อนแสงที่
นำเสนอนี้สัมพันธ์กับค่าบีอาร์ดีเอฟ
ของผ้าไหมมากกว่าแบบจำลองการ



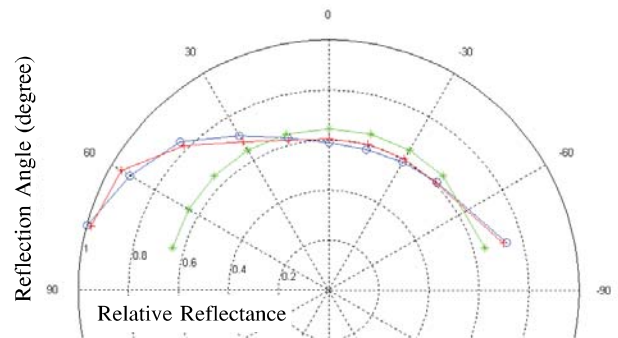
สะท้อนแสงอื่น และเมื่อนำมาเขียน
เส้นโค้งเชิงขั้วจะเห็นว่า เส้นโค้งของ
ค่าบีอาร์ดีเอฟที่ได้จากแบบจำลองที่

นำเสนอนี้ใกล้เคียงกับเส้นโค้งของ
บีอาร์ดีเอฟที่วัดจากผ้าไหม มากกว่าเส้น
โค้งของค่าบีอาร์ดีเอฟจากแบบจำลอง

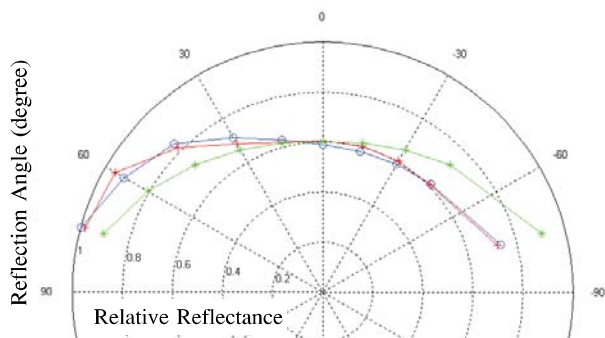
อื่น ดังแสดงใน รูปที่ ๑๒-๑๖



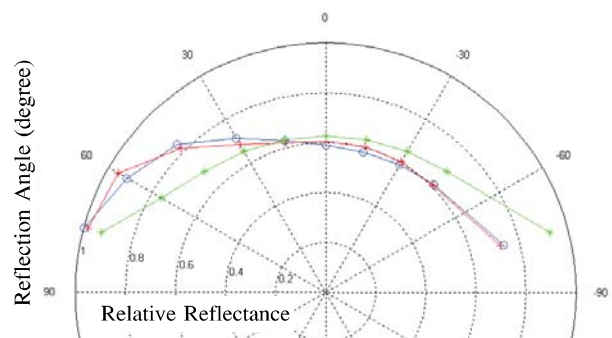
รูปที่ ๑๒ เส้นโค้งเปรียบเทียบระหว่างค่าการสะท้อนแสงที่วัดจากผ้าไหม (o) กับค่าการสะท้อนแสงที่คำนวณจากแบบจำลองที่นำเสนอของผ้าไหมตัวอย่างที่ ๓ ที่มุมตกกระทบ -๖๐ องศา



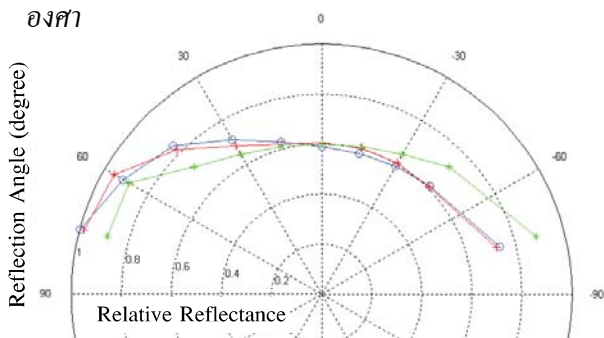
รูปที่ ๑๓ เส้นโค้งเปรียบเทียบระหว่างค่าการสะท้อนแสงที่วัดจากผ้าไหม (o) กับค่าการสะท้อนแสงที่คำนวณจากแบบจำลองที่นำเสนอ (+) และแบบจำลองฟอง (*) ของผ้าไหมตัวอย่างที่ ๓ ที่มุมตกกระทบ -๖๐ องศา



รูปที่ ๑๔ เส้นโค้งเปรียบเทียบระหว่างค่าการสะท้อนแสงที่วัดจากผ้าไหม (o) กับค่าการสะท้อนแสงที่คำนวณจากแบบจำลองที่นำเสนอ (+) และแบบจำลองโอเรนและนาร์ (*) ของผ้าไหมตัวอย่างที่ ๓ ที่มุมตกกระทบ -๖๐ องศา



รูปที่ ๑๕ เส้นโค้งเปรียบเทียบระหว่างค่าการสะท้อนแสงที่วัดจากผ้าไหม (o) กับค่าการสะท้อนแสงที่คำนวณจากแบบจำลองที่นำเสนอ (+) และแบบจำลองวาร์ด (*) ของผ้าไหมตัวอย่างที่ ๓ ที่มุมตกกระทบ -๖๐ องศา



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ตารางที่ ๑ ค่า RMS ของค่าการสะท้อนแสงจากแบบจำลองต่าง ๆ

ตัวอย่างที่	มุมตกกระทบ (องศา)	ค่า RMS ของ				
		แบบจำลอง ที่นำเสนอ	แบบจำลอง ของฟอง	แบบจำลอง ของ โอเรนและ นaylor	แบบจำลอง ของวาร์ด	แบบจำลอง ของ แอซิกมินและ เซอร์ลีย์
๑	๐	๐.๐๑๕๓	๐.๐๒๒๓	๐.๐๒๒๕	๐.๐๒๒๕	๐.๐๒๒๘
	-๑๕	๐.๐๑๑๒	๐.๐๑๖๗	๐.๐๑๒๔	๐.๐๑๕๓	๐.๐๑๗๔
	-๓๐	๐.๐๑๖๘	๐.๐๘๒๔	๐.๐๕๐๖	๐.๐๘๖๘	๐.๐๘๒๒
	-๔๕	๐.๐๒๕๖	๐.๑๑๐๐	๐.๑๓๑๔	๐.๑๑๖๓	๐.๑๐๖๗
	-๖๐	๐.๐๓๘๐	๐.๑๑๖๑	๐.๑๕๕๔	๐.๑๒๔๓	๐.๑๑๑๒
	-๗๕	๐.๐๑๕๔	๐.๐๕๒๗	๐.๑๓๑๕	๐.๐๕๗๕	๐.๑๐๐๘
๒	๐	๐.๐๔๐๕	๐.๐๐๗๓	๐.๐๔๐๕	๐.๐๒๘๓	๐.๐๔๐๕
	-๑๕	๐.๐๓๐๗	๐.๐๒๕๖	๐.๐๘๗๖	๐.๐๔๑๕	๐.๐๕๔๐
	-๓๐	๐.๐๑๐๐	๐.๐๔๘๗	๐.๑๑๖๕	๐.๐๖๐๕	๐.๐๖๕๔
	-๔๕	๐.๐๒๓๕	๐.๐๖๑๕	๐.๑๒๕๔	๐.๐๖๔๓	๐.๐๗๖๕
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	-๑๕	๐.๐๔๔๔	๐.๐๔๕๐	๐.๐๗๑๓	๐.๐๔๘๐	๐.๐๕๐๓
	-๓๐	๐.๐๑๐๕	๐.๐๗๐๗	๐.๑๐๕๗	๐.๐๗๒๘	๐.๐๖๘๕
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	-๖๐	๐.๐๒๑๔	๐.๐๕๑๘	๐.๑๖๑๐	๐.๑๐๓๖	๐.๐๘๔๓
	-๗๕	๐.๐๑๘๘	๐.๐๕๕๖	๐.๑๑๘๓	๐.๐๖๕๘	๐.๐๕๔๕

สรุป

งานวิจัยนี้ได้วัดและวิเคราะห์ลักษณะการสะท้อนแสงของพื้นผิวผ้าไหม พบว่า ผ้าไหมมีการสะท้อนแสงแบบผสม และมีสมบัติพิเศษที่สะท้อนแสงกล้าแบบ off-specular ผู้วิจัยได้

สร้างแบบจำลองสำหรับทดสอบการสะท้อนแสงของผ้าไหมขึ้น โดยปรับปรุงจากแบบจำลองการสะท้อนแสงของฟองและได้เปรียบเทียบกับแบบจำลองอื่น ๆ อย่างไรก็ตามแบบจำลองที่นำเสนอนี้ได้มาจากการ

วัดบีอาร์ดีเอฟเท่านั้น ดังนั้น การศึกษาเรื่องอิทธิพลของโครงสร้างผ้าไหม สมบัติเฉพาะของเส้นด้ายไหม จะช่วยให้สร้างแบบจำลองการสะท้อนแสงได้ถูกต้องและแม่นยำมากยิ่งขึ้น ซึ่งเป็นงานวิจัยในแนวลึกต่อไป



กิตติกรรมประกาศ

คณะผู้นิพนธ์ขอขอบพระคุณฝ่ายวิชาการ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) ที่ให้การสนับสนุนการวิจัยเรื่องนี้ ผ่านโครงการวิจัยเมธีวิจัยอาวุโส สกว. รหัสโครงการ RTA๕๐๘๐๐๐๔ นอกจากนี้ คณะผู้วิจัยขอขอบคุณศูนย์การวิจัยฟรอนเทียร์เมดิคัลเอนจิเนียริง (Research Center for Frontier Medical Engineering) แห่งมหาวิทยาลัยชิบะ ประเทศญี่ปุ่น ที่เอื้อเพื่อให้ใช้อุปกรณ์วิจัย ขอบพระคุณภาควิชาวิทยาศาสตร์ทางภาพถ่ายและเทคโนโลยีทางการพิมพ์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่เอื้อเพื่อค่าใช้จ่ายและค่า

เดินทางระหว่างประเทศ ซึ่งทำให้สามารถทำงานวิจัยบางส่วนที่ประเทศญี่ปุ่น

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Abstract

Measurement of Bidirectional Reflectance Distribution Function and Reflectance Modeling of Silk Fabrics

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Silk fabric is a fabric in which its gloss is perceived differently in comparison with other fabrics due to its specific reflectance. This research measured a Bidirectional Reflectance Distribution Function (BRDF) of three types of silk fabric having different weaving styles in conjunction with reflectance characteristics. Besides the measured BRDFs of the silk fabrics, its reflection model was then proposed. The proposed reflection model incorporates a specular reflection lobe and diffuse reflection lobe. The proposed model and the measured BRDFs of the three silk fabrics were fitted by the least-square error method. The measured BRDFs of the silk fabric were also fitted with Phong reflection model, Oren-Nayar reflection model and Torrance-Sparrow reflection. It was found that the proposed model fitted better with the measured BRDF than did the general models of Phong-reflection, Oren-Nayar reflection, and Torrance-reflection. The proposed model can thus be used to approximate, analyze and elucidate the characteristics of light reflection of the three silk fabrics.

Key words : BRDF, silk fabrics, reflection model

บทความที่ Under revision

หรือ Under review

**SYNTHESES, CHARACTERIZATION AND ANTIBACTERIAL
ACTIVITY OF CHITOSAN GRAFTED HYDROGELS AND
ASSOCIATED MICA-CONTAINING NANOCOMPOSITE
HYDROGELS**

Journal:	<i>Journal of Applied Polymer Science</i>
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Keywords:	swelling, hydrogels, nanocomposites, organoclay, biocompatibility

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Review

**SYNTHESES, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY
OF CHITOSAN GRAFTED HYDROGELS AND ASSOCIATED MICA-
CONTAINING NANOCOMPOSITE HYDROGELS**

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SYNTHESES, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF CHITOSAN GRAFTED HYDROGELS AND ASSOCIATED MICA- CONTAINING NANOCOMPOSITE HYDROGELS

Abstract

Chitosan (CS) grafted poly[(acrylic acid)-*co*-(2-hydroxyethyl methacrylate)] (CS-*g*-poly(AA-*co*-HEMA)) at different molar ratios of AA and HEMA, and the associated nanocomposite hydrogels of CS-*g*-poly(AA-*co*-HEMA)/mica were synthesized by radical copolymerization. The grafting positions at the amino or hydroxyl groups in the CS were identified by Fourier transform infrared spectroscopy. CS-*g*-poly(AA-*co*-HEMA) hydrogels were intercalated in the mica and the amount of hydrogel insertion did not affect the spacing of the silicate layers in mica. The higher mica loadings produced a rougher surface of the nanocomposite hydrogel. The water absorbency of the CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels decreased with increasing levels of mica loading to a lower level than those of the CS-*g*-poly(AA-*co*-HEMA) hydrogels. Both CS-*g*-poly(AA) and CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels exhibited a higher antiproliferative activity against *Staphylococcus aureus* than did the neat CS hydrogel with CS-*g*-poly(AA) revealing a very pronounced minimum inhibition concentration (MIC) of 1.56 mg mL⁻¹. The extent of mica loading in the CS-*g*-poly(AA-*co*-HEMA) nanocomposite hydrogels did not affect the MIC (12.5 mg mL⁻¹).

Keywords: Chitosan; Acrylic acid; 2-hydroxyethyl methacrylate; Mica; Nanocomposite hydrogel; Antibacterial property

1. Introduction

Hydrogels are slightly crosslinked hydrophilic polymers that can swell and absorb at least 15 times of their dry weight. The relative density of hydrophilic groups and the degree of crosslinking of the hydrogel are the two main factors that affect the water absorbency of a hydrogel.¹ Chitosan (CS) has found wide applications in the biomedical field, as well as other fields, because of its interesting biological properties, such as biocompatibility, biodegradability, hemostatic activity and bacteriostatic effects. Recently, CS-based hydrogels have been extensively researched and used in a wide field of applications because of their biocompatibility, biodegradability, non-toxicity and antibacterial activity,² as well as that CS is a plentiful, renewable and relatively cheap material. The hydrogels derived from CS grafted with hydrophilic vinyl monomers, such as acrylic acid (AA) or itaconic acid (IA), showed an increased water absorbency compared to CS alone.³⁻⁵ The 2-hydroxyethyl methacrylate (HEMA) monomer has long been used in biomedical applications because it improves the graft copolymer biocompatibility.⁶ HEMA monomer is harmful in the dental application if it does not completely polymerize, because it induces DNA damage, apoptosis and cell-cycle delay by the production of reactive oxygen species. However, it was found that the presence of antioxidant and/or CS can decrease the genotoxicity of HEMA monomer.^{7,8} CS-*g*-poly(AA-*co*-HEMA) was reported to exhibit good cytocompatibility and hemocompatibility and thus possesses a high potential as an application for wound dressing.³ Suitable wound dressings normally possess an antibacterial activity for protecting the wound from infection, and have a water absorbency of around 25 - 32 times their dry weight so as to be able to remove secretion or pus from the wound.⁹ Bacterial infection is one of the main factors for the development of chronic wounds. Infected wounds may cause trauma and higher

treatment costs. Pathogens frequently associated with wound infections include *Staphylococcus aureus* (*S. aureus*), *Enterococcus* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter* sp., *Proteus mirabilis*, *Klebsiella pneumonia*, *Streptococcus* sp., and *Candida albicans*. *S. aureus* is the most commonly found human pathogens which cause wound infections, boils, pustules and other human skin infections.¹⁰ Infection by *S. aureus* is accounted for about 20% frequency of occurrence in burns, wounds, and surgical sites. Due to the above mentioned, any developed wound dressing materials should therefore possess an inhibitory effect on the most frequently found *S. aureus* in wounds.

Generally, the gel strength of a bio-absorbent is naturally rather weak and this unfortunately limits its useful applications. To improve the gel strength, thermal and inherently antibacterial properties of CS and/or grafted CS, clay minerals, such as montmorillonite,¹¹ rectorite^{12,13} and sepiolite,¹⁴ have been used as a filler incorporated into the CS to produce CS/nanocomposite hydrogels. Wang et al.¹⁵ found that the antibacterial activity against *S. aureus* of CS/rectorite nanocomposite film was better than that of the neat CS film. This result agreed with the research reporting that clay minerals alone did not destroy bacteria, but they could adsorb and kill bacteria when the antimicrobial materials were intercalated into silicate layers.¹⁶

Mica is a clay mineral known as a 2: 1 phyllosilicate and its general formula is $AM_{2-3}T_4O_{10}X_2$, where A is usually Na, K or Ca. The M can be Fe, Al and Mg; T is Si and/or Al and X is (OH), O, Cl and F.¹⁷ It is composed of two-dimensional layers of an edge-shared octahedral sheet that is fused to form two tetrahedrals by the shared oxygen ions of one octahedral sheet and two tetrahedral sheets.¹⁸ Mica showed the antimicrobial activity, but it has a tendency of cytotoxicity with a larger platelet size than that of montmorillonite.¹⁹ Nanosilicate platelet of montmorillonite has a low

cytotoxicity with antibacterial activity.^{19,20} However, only a few research reports on CS/mica nanocomposite hydrogels are available, leaving its potential suitability for many applications, including as a wound dressing material, unknown. Therefore, the synthesis and characterization of grafted CS/mica nanocomposite hydrogels was the main aim of this research. The effects of the reaction parameters on the morphology, water absorption and antibacterial activities of the resultant hydrogels were investigated.

2. Experimental

2.1 Materials

CS with a viscosity average molecular weight of $57,000 \text{ g mol}^{-1}$ and an 85% deacetylation degree was purchased from Seafresh Industry Public Co., Ltd. (Bangkok, Thailand). Industrial grade AA and HEMA monomers were kindly supplied by Thai Mitsui Specialty Chemicals Co., Ltd. (Bangkok, Thailand). *N*, *N*'-Methylenebisacrylamide (*N*-MBA) crosslinker and *N*, *N*, *N*', *N*'-tetramethylethylenediamine (TEMED) co-initiator were purchased from Fluka (Buchs, Switzerland). Ammonium persulphate (APS), purchased from Ajax (Australia), was used as the initiator. Swelling mica was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deionized water, used as the reaction medium, was obtained from an Elga Deionizer (Model LA611, Marlow international, Bucks, U.K.). Sodium ampicillin (drug grade) was purchased from T.P. Drug Laboratories (Bangkok, Thailand). *Staphylococcus aureus* (*S. aureus*) isolate from textile wastewater was provided by the National Center for Genetic Engineering and Biotechnology (NSTDA, Pathum thani, Thailand). Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA), used for the growth of *S. aureus* were from Difco, New Jersey, USA.

2.2 Syntheses

2.2.1 Synthesis of grafted CS hydrogels

CS (1 g) was added into 30 ml of 2% w v⁻¹ acetic acid solution in a 500-ml four-necked round-bottom flask with a mechanical stirrer (Ika RW 28, IKA® Werke GmbH & Co. KG, Staufen Germany), assembled with a half-moon shaped Teflon blade paddle and a condenser. The mixture was stirred for 30 min at room temperature and then heated at 60 ± 2°C under nitrogen gas which was fed constantly through the gas inlet at 10 ml min⁻¹ for 30 min. APS (0.01-0.15 g) was then added and stirred for 10 min, followed by the sequential addition of either AA or HEMA or both and *N*-MBA (0.01 - 0.15 g) to the desired final concentration and stirred for 5 min. Finally, TEMED was added (0.01 - 0.4 g) to the mixture and stirred at 250 rpm for another 60 min. The reaction was then cooled down to room temperature and adjusted to pH 8 by 1 M sodium hydroxide solution. The product was dewatered with acetone, cut into pieces of approximately 1 x 1 cm square and dried in an oven at 50°C for 24 h. The dried product was extracted with an excess amount of methanol in a Soxhlet apparatus for 24 h to remove the unreacted components and obtain the CS grafted polymer (e.g. CS-*g*-poly(AA-*co*-HEMA)). The extracted product was dried in the oven at 50°C for 24 h, milled and sieved through a 100 mesh sieve to obtain the hydrogel particles in the range of 50 - 100 mesh (200 - 400 micrometers).

2.2.2 Synthesis of CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels

The desired amount of mica was dispersed in the mixture of AA and HEMA monomers for 60 min by stirring at 250 rpm with a magnetic stirrer. CS (1 g) was dissolved in 30 ml of 2% v v⁻¹ solution of acetic acid, and then added into a 500-ml four-necked round-bottomed reaction flask equipped with the mechanical stirrer and a

condenser. The reactor was then immersed in a temperature controlled water bath, stirred at 250 rpm under a constant nitrogen gas fed through the gas inlet tube for 30 min before APS (0.1 g) was added and stirred for 10 min. The well-dispersed mica suspension was added into the solution of CS and APS, stirred at 250 rpm and heated to $60 \pm 2^\circ\text{C}$ under the nitrogen gas circulation for 30 min before *N*-MBA (0.1 g) was then added and stirred for 5 min. Finally, TEMED (0.4 g) was added and stirred for another 60 min. The polymerization product was cooled down and the product was obtained by the same procedure as described above for the grafted CS hydrogels.

2.3 Grafting ratio and efficiency of the grafted CS hydrogels by acid hydrolysis

The grafted CS hydrogel (0.5 g) was dissolved in 50 ml of 1 M HCl in a 125-ml Erlenmeyer flask which was assembled with a condenser and refluxed at 120°C for 2 h. After the acid hydrolysis, the remaining solid (grafted copolymer) was filtered, dried and weighed. The filtrate was neutralized to pH 7 by 1 M sodium hydroxide solution and then precipitated by ethanol. The precipitate was filtered and dried at room temperature for 24 h to obtain its dry weight. Finally, the filtrate from the precipitation step was evaporated to remove the residual solvent. The white solid was dried at room temperature and weighed. The remaining solid and precipitate after acid hydrolysis were investigated for functional groups by Fourier Transform Infrared Spectroscopy (FT-IR; model Tensor 27, Billerica, USA) using KBr pellets. The grafting ratio, grafting efficiency and homopolymer content were calculated from Equations (1) - (3):²¹⁻²³

$$\% \text{ grafting efficiency} = \frac{\text{Weight of remaining solid after acid hydrolysis} \times 100}{\text{Weight of homopolymer} + \text{Weight of grafted copolymer}} \quad (1)$$

$$\% \text{ grafting ratio} = \frac{\text{Weight of remaining solid after acid hydrolysis} \times 100}{\text{Weight of precipitate from ethanol}} \quad (2)$$

$$\% \text{ homopolymer} = \frac{\text{Weight of grafted CS hydrogel before Soxhlet extraction} - \text{weight of grafted CS hydrogel after Soxhlet extraction}}{\text{Weight of grafted CS hydrogel before Soxhlet extraction}} \quad (3)$$

2.4 Identification of functional groups of grafted CS hydrogels and CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels

The functional groups of mica, the grafted CS hydrogels and grafted CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels were characterized by FT-IR.

2.5 Characterization of the grafted CS hydrogels and CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels

The surface morphologies of the mica, and the grafted CS and grafted CS/mica nanocomposite hydrogels were photographed on a scanning electron microscope (SEM, model JSM-5410LV, Tokyo, Japan) without prior cross sectioning. Each sample was fixed on an aluminum stub and coated with gold. X-ray diffraction (XRD) patterns of the samples were recorded using Bruker AXS (model D8 Discover, Bruker AXS Munich, Germany) with Cu radiation, voltage of 40 kV, a current of 40 mA and scanned from 1 - 35° with a scanning rate of 0.02° min⁻¹. The transmission electron micrographs (TEM) were taken on JOEL (JEM-2100, Jeol Ltd., Tokyo, Japan).

2.6 The effect of the reagent contents on water absorbency of grafted CS hydrogels and CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels

The water absorbencies of the grafted CS hydrogel and the grafted CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels were carried out with distilled water at room temperature in a closed system to minimize water evaporation. Distilled water (200 ml) was added to 0.1 g of the dry grafted hydrogels or the nanocomposite hydrogels. The hydrogel was swollen to equilibrium for 24 h and the swollen gel was then filtered through a 100-mesh aluminum screen sieve for 2 h. The water absorbency was determined for at least three replications for each synthesized product by Equation (4):

$$\text{Water absorbency } (Q) = \frac{(W_s - W_d)}{W_d} \quad (4)$$

where W_s is the weight of swollen hydrogels or nanocomposite hydrogels (g) and W_d is the weight of the dry hydrogels or nanocomposite hydrogels (g).

2.7 Antibacterial activity

2.7.1 Turbidity method

The CS, CS-*g*-poly(AA), CS-*g*-poly(HEMA) and CS-*g*-poly(AA-*co*-HEMA) hydrogels, and the CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels, each weighing 250 mg, and distilled water were sterilized by UV radiation for 1 h prior to the test.

The diluted stock culture of *S. aureus* was adjusted to 0.5 units by sterile deionized water. The *S. aureus* suspension (2.5 ml) was inoculated into 47.5 ml of sterile Mueller-Hinton Broth (MHB) in a 250 ml sterile Erlenmeyer flask. For each hydrogel type, the sterile sample (250 mg) was then added to the *S. aureus* suspension in MHB media and incubated at 37°C while being shaken at 250 rpm. At every two hours a 50 ml aliquot was harvested and the swollen gel removed by filtration through

filter paper no. 1 (Schleicher & Schuell, Whatman, Kent, U.K.) prior to measuring the OD_{610 nm} of the filtrate as a measure of the growth of *S. aureus* using a spectrophotometer (Spectronic 21, Bausch & Lomb Inc., Rochester, New York, U.S.A). The OD results of the growth with or without the hydrogel were plotted versus time to obtain the bacterial growth profiles. The culture time at which the antibacterial activity of the grafted CS hydrogel was found to be maximal by the optical density measurement was used to evaluate the total replication competent (viable) bacterial number using the total plate count method (see section 2.7.2. below) so as to calculate the mean colony-forming units per milliliter (CFU ml⁻¹), and from this the relative degree of inhibition of bacterial proliferation can be obtained.

2.7.2 Total plate count method

The six and eight hour old *S. aureus* cultures in MHB were diluted with sterile deionized water to 10⁸ - 10⁹ CFU ml⁻¹ and then 100 µl aliquots of each diluted *S. aureus* suspension were spread on each MHB plate. The plates were incubated for 24 h at 37°C where upon the number of viable bacteria was counted. Mean CFU ml⁻¹ and the relative inhibition were calculated by Equations (5) and (6), respectively. The negative control was the sterilized *S. aureus* cultured in MHB.

$$CFU / ml = \left(\frac{\text{mean numbers of counted colonies}}{\text{amount of dilute } S.aureus \text{ for spread plate (ml)}} \right) \times \text{dilution factor} \quad (5)$$

$$\text{Relative inhibition} = 100 - \left(\frac{CFU / ml \text{ of the sample}}{CFU / ml \text{ of negative control}} \right) \times 100 \quad (6)$$

2.7.3 Minimum inhibition concentration (MIC)

The antibacterial activity was determined in terms of the minimum inhibition concentration (MIC). The diluted stock culture of *S. aureus* was adjusted to 0.5 A₆₁₀ units by sterile deionized water and 100 µl was inoculated into 4.9 ml of sterile MHB in a 10 ml test tube. The hydrogels, each evaluated at the five concentrations of 0, 3.12, 6.25, 12.5 and 25 mg ml⁻¹, were added to the 0.01 A₆₁₀ unit suspensions of *S. aureus* (see above) in sterile MHB. The cultures were then incubated at 37°C with shaking at 120 rpm for 24 h. The mean CFU ml⁻¹ of each sample tested were measured by the total plate count method as above and the experiment was performed in triplicate. The MIC was thus determined from the derived CFU ml⁻¹ and statistical significance (ANOVA) was accepted at the 99% significant level or $\alpha = 0.01$.

3. Results and discussion

3.1 Characterization of the grafted hydrogels

3.1.1 Grafted CS hydrogels

The spectrum of the CS hydrogel (Figure 1(a)) shows the characteristic amino group peaks at 3428 cm⁻¹ for N-H stretching, 1640 cm⁻¹ for the C=O stretching of amide I,²⁴ 1594 cm⁻¹ for the N-H bending of amide II, 1381 cm⁻¹ for the -NHCO stretching of amide II²⁵ and 1253 cm⁻¹ for C-N stretching.²⁶ The characteristic peaks of the saccharide structure in CS exhibit at 1152 cm⁻¹ for the bridge O stretching,^{23,27} and 1085 cm⁻¹ and 1031 cm⁻¹ for the C₃-OH and C₆-OH stretching, respectively.²⁵ In addition, the absorption peak at 1420 cm⁻¹ is the O-H and C-H vibration which indicates the presence of a hydroxyl group.²⁴ The peak of C-H stretching appears at 2921 and 2864 cm⁻¹.²⁷

The CS-*g*-poly(AA) hydrogel spectrum (Figure 1(b)) shows a new absorption peak at 1724 cm^{-1} assigned to C=O stretching of the carboxyl group from the grafted poly(AA) part,²⁷ which was shifted from the peak at 1713 cm^{-1} of the carboxyl group of poly(AA). In addition, the new absorption peaks at 1563 cm^{-1} and 1402 cm^{-1} are ascribed to asymmetric and symmetric stretching of the carboxylate anion of poly(AA) in the CS-*g*-poly(AA) hydrogel.³ The absorption peak at 1594 cm^{-1} , assigned to N-H bending of CS, disappeared in the CS-*g*-poly(AA) hydrogel. Thus, the NH_2 group is grafted in the CS backbone.²⁸ Moreover, the characteristic peaks of the saccharide structure of CS in the CS-*g*-poly(AA) hydrogel was shifted to 1065 cm^{-1} and 1019 cm^{-1} ($\text{C}_3\text{-OH}$ and $\text{C}_6\text{-OH}$ stretching) and the peak of O-H bending at 1420 cm^{-1} disappeared (Figure 1(b)). From these results, the poly(AA) chains were confirmed to be grafted at both the NH_2 and OH sites on the CS backbone.

The CS-*g*-poly(HEMA) spectrum (Figure 1(c)) shows an absorption peak at 1730 cm^{-1} , assigned to C=O asymmetric stretching, which indicates the presence of the ester carbonyl group of poly(HEMA),³ and a new absorption peak at 746 cm^{-1} that is attributed to the CH_2 rocking of the poly(HEMA) moiety.²⁹ The absorption peak of N-H stretching of CS also disappeared. Thus, the NH_2 group of CS had been reacted in the graft polymerization.²⁸ The characteristic peaks of the saccharide structure of CS in the CS-*g*-poly(HEMA) hydrogel were shifted to 1069 cm^{-1} and 1021 cm^{-1} ($\text{C}_3\text{-OH}$ and $\text{C}_6\text{-OH}$ stretching) and the CS O-H bending peak at 1420 cm^{-1} disappeared. Thus, the poly(HEMA) was grafted at both NH_2 and OH sites on the CS backbone as well.

The CS-*g*-poly(AA-*co*-HEMA) hydrogel spectrum (Figure 1(d)) shows the absorption peak at 1722 cm^{-1} that is assigned to the C=O asymmetric stretching and so indicates the presence of the ester carbonyl group of the poly(HEMA) moiety,³ and

a new absorption peak at 753 cm^{-1} that is attributed to the CH_2 rocking of the poly(HEMA) moiety.²⁹ In addition, it also shows peaks at 1585 cm^{-1} ($\text{C}=\text{O}$ asymmetric stretching) and 1402 cm^{-1} ($\text{C}=\text{O}$ symmetric stretching) of the carboxylate anion in the poly(AA) moiety.³ Moreover, the characteristic peaks of the saccharide structure of CS in the CS-g-poly(AA-co-HEMA) hydrogel (Figure 1(d)) shifted to 1074 and 1021 cm^{-1} ($\text{C}_3\text{-OH}$ and $\text{C}_6\text{-OH}$ stretching) and the peak of O-H bending at 1420 cm^{-1} (Figure 1(a)) disappeared (Figure 1(d)). Thus, poly(AA-co-HEMA) was also grafted at both the NH_2 and OH sites on the CS backbone.

3.1.2 CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels

The FT-IR spectrum of mica is shown in Figure 1(e). It shows a peak of O-H stretching at $3600 - 3400\text{ cm}^{-1}$ that indicates the presence of the hydroxyl group. The absorption peaks at 2924 and 2852 cm^{-1} are attributed to the C-H stretching of the organic part and indicate that the mica used is synthetic mica. It also shows the absorption peaks of H-O-H bending of water at 1637 and 1620 cm^{-1} ,³⁰ and Si-O and Al-O stretching peaks at 1016 and 473 cm^{-1} .³¹

The FT-IR spectra of CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels, containing mica at 7, 10 or 15% w w⁻¹ of the monomer, are shown in Figure 1(f-h), respectively. In comparison with the spectra of mica (Figure 1(e)), the characteristic peaks of CS are shifted to 1636 and 1389 cm^{-1} , whilst the characteristic peaks of poly(AA-co-HEMA) are also shifted to 1724 , 1572 , 1404 and 754 cm^{-1} . In addition, new absorption peaks appear at 473 , 469 and 467 cm^{-1} (Figure 1(f-h), respectively), all of which are attributed to the Al-O stretching of mica. The intensity of the absorption peak at 1016 cm^{-1} (Si-O stretching) increased with increasing mica loadings in the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels, which

indicates the existence of mica in the CS-*g*-poly(AA-*co*-HEMA) nanocomposite hydrogel. Based on these FT-IR data, the AA or HEMA group is grafted at the OH group of the C-6 position and/or the amino group of CS. The FT-IR peaks in the spectra further confirmed that CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels with various loadings of mica were successfully synthesized.

3.2 The grafting performance of grafted CS hydrogels

Analysis of the acid hydrolysis suggests the grafting ratio of the CS-*g*-poly(AA) hydrogel (87%) is lower than that of the CS-*g*-poly(AA-*co*-HEMA) (269%) and CS-*g*-poly(HEMA) hydrogels (343%). However, the grafting ratios of CS-*g*-poly(AA-*co*-HEMA) and CS-*g*-poly(HEMA) hydrogels were calculated from the weight differences because a poly(HEMA) C=O peak at 1710 and 1722 cm⁻¹ is still observed. Thus, at least some poly(HEMA) is still incorporated in the precipitate after acid hydrolysis, it means that the acid hydrolysis of the CS-*g*-poly(HEMA) was not completed. In comparison with the amount of CS, a greater amount of HEMA was used and so HEMA can generate an interpenetrate polymer network (IPN) hydrogel³² on the CS backbone. This result reflects the high grafting ratio as expected. The grafting efficiency of CS-*g*-poly(AA), CS-*g*-poly(AA-*co*-HEMA) and CS-*g*-poly(HEMA) hydrogels is 93, 94 and 96%, respectively. The amount of homopolymer normally generated during graft polymerization and the homopolymer chains could form the IPN chains. Therefore, the amount of homopolymer cannot be derived directly just from the Soxhlet extraction and acid hydrolysis. Therefore, the amounts of free poly(AA), poly(AA-*co*-HEMA) and poly(HEMA) found are only 3, 5 and 3%, respectively, which are considered to be quite small, and would probably have been incorporated as the IPN in the polymeric hydrogels.

3.3 Surface morphology

The surface morphologies of the CS, mica, CS-*g*-poly(AA-*co*-HEMA) hydrogel and CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels with 5 to 15% (w w⁻¹ of the monomer) mica loadings are shown as the representative SEM micrographs in Figure 2. The relatively smooth surface of the CS hydrogel and the flat sheets of mica at approximately 1 - 3 micrometers along with their aggregated state is clearly visible (Figure 2(a,b)). The surface of the CS-*g*-poly(AA) hydrogel (see Figure 2(c)) exhibits the larger porous structure in many areas than those of CS-*g*-poly(HEMA) (see Figure 2(d)) and CS-*g*-poly(AA-*co*-HEMA) hydrogel (see Figure 2(e,f)). The surface morphology of the CS-*g*-poly(HEMA) hydrogel shows a gel network surrounded by a wide spread of uniformly distributed micro-pores and this is especially regular on the surface of the CS-*g*-poly(AA-*co*-HEMA) hydrogel (Figure 2(d)). The CS-*g*-poly(AA-*co*-HEMA) hydrogel surface exhibits further a nano-porous structure (Figure 2(e,f)).

The internal morphologies of the CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposites containing 5 - 15% w w⁻¹ mica of the monomer (Figure 2(g-j)) are slightly different from those of the mica-free CS-*g*-poly(AA-*co*-HEMA). It is seen that the higher the mica loading, the more the surface roughness increased and that the mica flakes are imbibed in the inner areas of the gel. When the mica loadings were high, they were agglomerated, aggregated, resided, deposited and covered the whole polymer surface. Based on these results, CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels are confirmed to have been successfully synthesized. One must state that no residual HEMA was retained in the resulting hydrogels because no indication of the double bonds peaks was found in the IR spectra because many extractions of the unused materials were made in solvent.

3.4 XRD and TEM analyses

The XRD pattern of the CS hydrogel (Figure 3(a)) shows the 2-theta of CS at 10.53° and 19.93° , which corresponds to the hydrated crystals of low crystallinity of the form I and crystallinity of the form II, respectively.^{33,34} The XRD pattern of CS-g-poly(AA), CS-g-poly(HEMA) and CS-g-poly(AA-co-HEMA) exhibit the broader and weaker peaks at 20.19° , 18.49° and 20.31° , respectively, whereas the 2-theta at 10.53° of all these three CS grafted hydrogels disappeared. These indicated that the crystallinity of the grafted CS was dramatically decreased. Changes in the intensity of the peaks at approximately 20° relate to different packing of the chains and/or different hydrogen bonding networks in the grafted CS.³⁵ Thus, the introduction of AA and/or HEMA chains onto the surface of the CS causes the breakdown of its crystallinity. These results confirmed that the grafting of polymer onto the CS chain occurred.^{33,34,36} The XRD patterns of mica revealed 2-theta values of 7.13° and 9.22° that correspond to a basal spacing of 13.58 and 9.58 Å, respectively. The XRD patterns of the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogel reveal that the 2-theta value of mica in the nanocomposite hydrogels were shifted towards a lower angle (6.27°) and that the interlayer spacing of mica increased by 1.7 Å compared with that of the swelling mica. This confirms that intercalation had taken place and that the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels are intercalated composites with more spacing in the silicate layers.³⁷ In addition, it also shows that increasing the mica loadings from 5 to 15% (w w⁻¹ based on the weight of monomer) does not affect the intercalated structure of the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels but rather they exhibit the same basal spacing. These results agree well with the previous report of Wang and coworkers.¹²

Representative TEM micrographs of the CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels with mica loadings of 5, 7, 10 and 15% w w⁻¹ of the monomer are shown in Figure 4. The dark lines in Figure 4 belong to the mica structure and the bright area is the grafted chitosan matrix. All the TEM micrographs of nanocomposite hydrogels revealed that the distance between the two adjacent dark lines, the spacing of the two silicate layers, results in an intercalated structure. Indeed, parts of the silicate layer can be seen in Figure 4(a-d). The results of the TEM analysis are consistent with the XRD data (Figure 3), and support that CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposite hydrogels have an intercalated structure and no significant if any level of an exfoliated structure.

3.5 Equilibrium water absorbency

3.5.1 Effect of monomer type (AA or HEMA) on the properties of the grafted CS hydrogels

The equilibrium water absorbency of CS alone was rather low but was significantly affected by the copolymerization with AA, but not with HEMA (Table 1). CS-*g*-poly(AA) had ten-fold higher water absorbency, whereas the water absorbency of the CS-*g*-poly(HEMA) was not significantly different to that for CS alone (Table 1). The water absorbency of the CS-*g*-poly(AA-*co*-HEMA) decreased ~1.2- and 3.2-fold as the AA: HEMA ratios decreased from 3:1 to 1:1 and 1:3, respectively (Table 1). That the water absorbency of CS-*g*-poly(AA-*co*-HEMA) increased as the content of AA increased is due to the increase in the density of hydrophilic groups from the carboxylic acid contents,³⁸ which then provides a greater repulsion of the anionic portion allowing for the expansion of the polymer chains. This also then explains the increased water absorbency of the CS-*g*-poly(AA)

hydrogels over that of the CS hydrogels. On the other hand, CS-*g*-poly(HEMA) had very low water absorbency because HEMA is not as polar as AA but rather has a rather rigid chain due to the methacrylate substituent group. The longer chain of the poly(HEMA) hydrogel thus is likely to have resulted in more molecular entanglements that limited the chain expansion³⁹ and caused the water absorbency of the CS-*g*-poly(HEMA) hydrogel to decrease. Thus, although CS-*g*-poly(HEMA) would be unlikely to be beneficial to intercalate with mica, the HEMA moiety is more compatible with human cells than the other types of monomer moieties.

3.5.2 Effect of mica loading

The equilibrium water absorbency of the CS-*g*-poly(AA-*co*-HEMA) hydrogel at a 1:1 AA: HEMA molar ratio was ~5.4- and 5-fold higher than that of CS or CS-*g*-poly(HEMA), respectively, but almost half that of the CS-*g*-poly(AA) hydrogel (Table 1). The addition of mica at 5, 7 or 10% (w w⁻¹ of monomer) caused a 1.2-fold drop in the equilibrium water absorbency from that seen with the mica-free CS-*g*-poly(AA-*co*-HEMA) hydrogel, but did not vary significantly between the three different mica loading levels (Table 1). In contrast, when the mica loading was increased to 15% w w⁻¹ of monomer, the water absorbency was significantly decreased a further 1.3-fold compared to that seen with the 5 – 10% w w⁻¹ mica loaded CS-*g*-poly(AA-*co*-HEMA), or 1.54-fold less than that for the mica-free CS-*g*-poly(AA-*co*-HEMA). This is because the polymer chains were inserted into the spacing between the silicate layers of mica and so the polymer chains were restricted in these limited spaces and so less able to expand. Another reason could be the degree of rigidity of the nanocomposite hydrogel that is induced by the mica. This also supports the notion that the structure of this nanocomposite hydrogel was intercalated.

3.6 Antibacterial activity

Particle sizes of the CS, CS-g-poly(AA-co-HEMA) hydrogels and CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogel were in the range of 200 - 400 μm by the sieve size 50-100 mesh which is anticipated to have an important effect on the bacterial growth inhibition.⁴⁰ This was therefore evaluated using a co-culture of *S. aureus* with each hydrogel type and evaluating the bacterial growth relative to the control culture by measuring the culture turbidity by absorbance (after removal of the hydrogel by filtration), and by total plate counts of replication competent ("viable") cells, over a 24 h time period.

3.6.1 Turbidity method

This well used method assumes that the turbidity of a medium solution increases solely due to the increase in the number of bacterial cells. Although this method is easy to perform the bacteria sample must be in a liquid suspension state, and typically it can only be used to detect bacteria in the range of 10^6 to 10^7 bacteria mL^{-1} . Moreover, the measurement of bacterial growth in the lag phase is less accurate and is an indirect measurement that needs a calibration curve, where a direct measurement of bacterial counts must be performed.

The preliminary results of the antibacterial activity testing of the CS-g-poly(AA-co-HEMA) hydrogel, evaluated by measuring the optical density at 610 nm every 2 h and compared with the negative control (containing only the medium and the gram-positive *S. aureus*), revealed three phases in the growth cycle (Figure 5). The lag phases of *S. aureus* growth in the negative control and in the presence of CS-g-poly(AA-co-HEMA) hydrogel were similar, lasting for 4 h. Then, during the exponential phase, the growth rate of *S. aureus* grown in MHB containing the CS-g-

poly(AA-co-HEMA) hydrogel was lower than that of the negative control during the first 2 h of growth (4 - 6 h of total culture time), but then this growth rate decreased in the control culture from after 6 h up to reaching the start of the stationary phase (12 h), whereas the growth rate did not slow down in the cultures with CS-g-poly(AA-co-HEMA) until 10 - 12 h of total culture time, by which time the total bacterial number (as culture optical density) was slightly higher (1.5-fold) than in the control culture. From 12 – 24 h of total culture time, *S. aureus* in both conditions remained in stationary phase. Thus, the presence of the CS-g-poly(AA-co-HEMA) hydrogel appeared to render a slower early exponential growth rate of *S. aureus* (or to delay it) but did not reduce the final bacterial density reached after 12 – 24 h in the stationary phase. Since we did not count the viable bacteria that are attached to the gel, or wash them out from the gel, then the CS-grafted hydrogels may not be inhibitory at all but rather the bacteria grow attached to the gel surface and so are not in suspension first and only in suspension subsequently after the gel surface is full swollen and thus has the apparent delayed kinetics appearance.

3.6.2 The total Plate count method

The antibacterial activities of sterile sodium ampicillin (positive control), CS and grafted CS hydrogels against *S. aureus* were determined by the total plate count method. The mean CFU ml⁻¹ and the relative inhibition of CS and grafted CS hydrogels are summarized in Table 2. The relative inhibition observed with the CS hydrogel was 2.9-fold greater at 6 h of culture time (early log phase) than that after 8 h of culture (the late log phase). When the CS was grafted with poly(AA), the antibacterial activity was significantly improved 1.53- and 4.23-fold after both 6 and 8 h of culture at 93 and 89% relative inhibition, respectively, because of the

hydrophilicity of poly(AA),⁴¹ and the biological activity of the free amino groups at the C-2 position of the native CS.⁴² The higher antibacterial activities of the CS-g-poly(AA) hydrogel can be caused by the interaction between the negative charges on the bacteria surface with the positive surface of the CS-g-poly(AA). The more positively charged hydrogels could promote bacterial adhesion onto the hydrogel surface and reduce the remaining amount in the free suspension, resulting in a decrease in the bacteria colonies in the liquid medium. It is relevant to note here that the amount (molar ratio) of the AA or HEMA monomers that were grafted onto the CS is almost the same for the three different grafted CS derivatives studied here.

It was anticipated that the pores on the CS-g-poly(AA) hydrogel surface would improve the ability to capture bacteria. Although, the relative inhibition of the CS-g-poly(AA-co-HEMA) hydrogel would be higher than that of CS, because of the hydrophilicity resulting from the carboxyl group of poly(AA), the surface of the CS-g-poly(AA-co-HEMA) hydrogel is much rougher than the ungrafted CS hydrogel surface, which may increase bacterial adhesion sites and result in a higher antibacterial activity in the CS-g-poly(AA-co-HEMA) hydrogel than that of the CS. However, the relative inhibition of the CS-g-poly(AA-co-HEMA) hydrogel was found to actually be 1.35- and 3.3-fold lower than that of the CH-g-poly(AA) hydrogel in the early (6 h) and late (8 h) log phases, respectively. This is likely to be due to the decreased hydrophilicity of the HEMA moiety.⁴¹ Thus, graft copolymerization of CS with a 1:1 molar ratio of AA: HEMA can delay (or inhibit) the growth of *S. aureus* in the early exponential phase.

The antibacterial activity of the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels are summarized in Table 2. The relative inhibition of CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels having mica loadings at 7 and

10% w w⁻¹ of the monomer was slightly higher (1.3- and 1.11-fold) at a culture time of 6 h than that of the CS and CS-g-poly(AA-co-HEMA) hydrogel, respectively. This finding is in agreement with those reported by Wang et al.¹⁵ However, CS-g-poly(AA-co-HEMA) exhibited higher relative inhibition against *S. aureus* growth by 1.2- and 1.1-fold comparing with 10 and 7% w w⁻¹ mica loading, respectively, at 8 h of culture. Increasing the mica loading to 15% w w⁻¹ of the monomer significantly decreased the apparent antibacterial activity 2.03- and 2.13-fold of that seen with no mica addition, and 2.25- and 1.9-fold of that seen with a 7% w w⁻¹ mica loading after 6 and 8 h of culture, respectively. This result agreed quite well with Meng et al.,¹⁶ finding that CS intercalated mica hydrogels could kill more bacteria than the neat CS or the clay minerals.

Nanocomposite hydrogels can generate a larger surface area to absorb more bacterial colonies and so give a greater chance to increase the antibacterial activity through interactions between the positive charges of the CS hydrogel and the negative charges on the cytoplasmic cell membrane of the bacteria.^{15,42} In addition, *S. aureus* does not have an outer membrane to prevent the influx of foreign molecules⁴² and so it can be inhibited more easily. However, the higher mica loading at 15% w w⁻¹ of the monomer revealed a significantly decreased level of relative inhibition of *S. aureus* growth. This might be because the higher mica content screens and prevents the polymer chains from adhering with the bacteria to trigger the antibacterial activity.

3.6.3 Minimum inhibition concentration (MIC)

The MIC obtained for the CS-g-poly(AA) at 1.56 mg ml⁻¹ against the 0.01 A₆₁₀ Units of *S. aureus* cultured in MHB for 24 h (i.e. to the late stationary phase) at room temperature was 16- and >16-fold better than that of CS and CS-g-poly(HEMA),

respectively (Table 3). These MICs agree with that of viable counts and relative inhibition of *S. aureus* in the log phase of growth (Table 1). The extent of the water absorbency and acidity of the hydrogels plays influencing roles as described in the total plate count method for the MIC result. The more the hydrogel expanded, the higher the number of colonies of *S. aureus* that could be trapped and inhibited through the diffusion effect by water in the hydrogel. The higher acidic moiety of the CS-g-poly(AA) hydrogels, which carry a higher positive charge, may enhance the inhibitory effect with CS. It is worth noting that the presence of the COO^- group in the poly(AA) containing hydrogels (CS-g-poly(AA) and CS-g-poly(AA-co-HEMA)) becomes a major driving force for bacterial adhesion in the positively charged CS. AA in the feed is a key factor governing the antibacterial property in the resulting hydrogel and nanocomposites hydrogels. The high MIC value for the CS-g-poly(HEMA) was potentially caused by the proposed semi-IPN structure and the rigid conformation of poly(HEMA) chains grafted onto the CS backbones.

Surprisingly, the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels with mica loadings of 5, 7, 10 and 15% w w⁻¹ of the monomer had the same MIC values as each other and as that for the CS-g-poly(AA-co-HEMA) hydrogel (Table 3). This correlates with the almost constant water absorbency observed for the CS-g-poly(AA-co-HEMA) and the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels regardless of the mica loadings but relatively lower water absorbency at 15% w/w. Likewise, a much higher concentration of CS (25 mg ml⁻¹) or CS-g-poly(HEMA) (>25 mg ml⁻¹) or CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels (12.5 mg ml⁻¹) is required for inhibiting *S. aureus* after 24 h of incubation.

Typical SEM micrographs of *S. aureus* cells cultured for 24 h in the MHB media alone (negative control), and in the various grafted CS copolymer and CS-g-

poly(AA-co-HEMA)/mica nanocomposite hydrogels are shown in Figure 6. Since the positive control in the total plate count did not produce any viable cells, then the positive control in MIC was not performed. The normal roundish-shapes of *S. aureus* cells were evident when cultured in the MHB media alone and also as adhered on the surface of mica (Figure 6(a,b), respectively). However, the cell walls of some of the *S. aureus* in or on the CS-g-poly(HEMA) and CS-g-poly(AA-co-HEMA) hydrogels after culture for 24 h were destroyed to give open cells (Figure 6(c) as shown in Figure 6(d)). Moreover, of the *S. aureus* cells that were adsorbed and immobilized on the surface and in the pores of the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels, some had distorted shapes and were destroyed to give fractured cell walls and these were randomly distributed in the hydrogel matrix (Figure 6(e-h)).

Although the water absorbency of the nanocomposite hydrogels was relatively lower than that of the grafted CS hydrogel, the hydrogels had larger surface area to absorb more bacterial colonies.¹⁵ With this compensation, the MIC values of nanocomposite hydrogels are not different from that of the grafted CS hydrogel. Thus, CS-g-poly(AA-co-HEMA) hydrogel or CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels were at an equilibrium condition and gave similar MIC values.

The MIC values and SEM micrographs support that the mica content in the grafted CS hydrogel did not influence the antibacterial activity. On the other hand, the different results from the plate count method are caused by the absorbance assays undertaken in the mid- and late-log phases of growth at 6 and 8 h, respectively, whilst the MIC was evaluated at a late stationary phase (24 h). The different culture times could have resulted in different cell numbers due to different chances of cell adsorption by the nanocomposite hydrogels, as well as growth and death rates

4. Conclusion

CS was used to graft with AA and/or HEMA to give the grafted-CS hydrogels. CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels were synthesized at different mica loadings and all hydrogels were characterized by FT-IR spectroscopy, SEM, XRD, TEM and equilibrium water absorbency. FT-IR spectroscopy indicated that the CS was grafted at the NH_2 and OH sites on the CS backbone. The water absorbency of the CS-g-poly(AA) hydrogel was the highest whilst those of CS and CS-g-poly(HEMA) were the lowest. The CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels had an intercalated structure, as revealed by XRD and TEM analyses.

At the fixed 1:1 molar ratio of AA: HEMA, the extent of water absorbency of the nanocomposite hydrogels was constant at 5, 7 and 10% (w w^{-1} of the monomer) mica loading but slightly decreased at 15% w w^{-1} mica loading. The CS-g-poly(AA) hydrogel can inhibit the early-to-mid-log phase growth of *S. aureus* up to 93% with a MIC value as low as 1.56 mg ml^{-1} . For the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels, the relative inhibition was very similar (at ~81%) with mica loadings of 5, 7 and 10% w w^{-1} of the monomer, but abruptly decreased at a 15% w w^{-1} mica loading. Regardless of the mica loading levels in the CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels, the MIC value at the late stationary phase was constant at 12.5 mg ml^{-1} . The CS-g-poly(HEMA) had a greater MIC ($>25 \text{ mg ml}^{-1}$) than CS (25 mg ml^{-1}). Several possible explanations for the different inhibitory activities against *S. aureus* are discussed, but await further research for clarification.

Acknowledgements

The authors would like to acknowledge the financial support from the Thailand Research Fund under the Research Team Aided Program Contract no. RTA5080004. Partial financial support from the Graduate School, Chulalongkorn University is acknowledged. The provision of research facilities by the Imaging Polymer Laboratory of Chulalongkorn University's Department of Imaging and Printing Technology, Faculty of Science is highly appreciated. English edition by the Publication Counselor Unit of the Research Division, Faculty of Science, Chulalongkorn University, and fruitful suggestion provided by Dr. Robert Butcher are highly appreciated.

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Table 1. The water absorbency of hydrogels derived from CS, AA and /or HEMA grafted-CS copolymers and CS-g-poly(AA-*co*-HEMA)/mica nanocomposites.

Polymer	AA: HEMA (mol ratio)	Mica (w w ⁻¹ of monomer)	Water absorbency (g g ⁻¹) ¹
CS	--	--	10 ± 2
CS-g-poly(AA)	--	--	101 ± 4
CS-g-poly(HEMA)	--	--	11 ± 1
CS-g-poly(AA- <i>co</i> -HEMA)	3:1	--	64 ± 2
	1:1	--	54 ± 3
	1:3	--	20 ± 2
	1:1	5	45 ± 3
	1:1	7	44 ± 1
	1:1	10	46 ± 2
	1:1	15	35 ± 5

¹Data are shown as the mean ± 1 SD and are derived from 5 repeats. Means within the column are significantly different (p<0.05).

Table 2. Total plate counts (replication competent cells) and the relative inhibition of growth of *S. aureus* in the presence of sodium ampicillin and the hydrogels of CS, AA and/or HEMA grafted-CS copolymers and CS-g-poly(AA-co-HEMA)/mica nanocomposites with various mica loadings.

Sample type	Mica (% w w ⁻¹)	6 h culture time		8 h culture time	
		CFU ml ⁻¹ (x 10 ⁹) ¹	Relative inhibition (%)	CFU ml ⁻¹ (x 10 ⁹) ¹	Relative inhibition (%)
Negative control ²	--	6.95 ± 1.51	-	8.15 ± 1.25	-
Positive control ³	--	0	100	0	100
CS ⁴	--	2.7 ± 0.25	61	6.4 ± 1.68	21
CS-g-poly(AA) ⁴	--	0.52 ± 0.48	93	0.91 ± 0.85	89
CS-g-poly(HEMA) ⁴	--	2.18 ± 0.28	69	5.95 ± 1.25	27
CS-g-poly(AA-co-HEMA) ⁴	--	1.90 ± 0.19	73	2.92 ± 0.07	64
CS-g-poly(AA-co-HEMA) ⁴	7	1.4 ± 0.37	81	3.6 ± 0.55	57
CS-g-poly(AA-co-HEMA) ⁴	10	1.5 ± 0.34	81	3.9 ± 0.31	54
CS-g-poly(AA-co-HEMA) ⁴	15	5.0 ± 1.68	36	5.9 ± 2.4	30

¹CFU ml⁻¹ is shown as the mean ± 1 SD and are derived from 6 repeats. Means within a column or across a row are significantly different (p<0.05).

²Negative control contains only the MHB medium and *S. aureus*.

³Positive control contains 250 mg of the sodium ampicillin, MHB medium and *S. aureus*.

⁴All hydrogel samples were co-cultured with *S. aureus* in MHB media at 250 mg ml⁻¹.

Table 3. Minimum inhibition concentration of hydrogels derived from CS, AA and/or HEMA grafted-CS copolymers and CS-*g*-poly(AA-*co*-HEMA)/mica nanocomposites with various mica loadings.

Sample test	Mica (% w w ⁻¹)	CFU ml ⁻¹ (x 10 ⁸) ¹	MIC (mg ml ⁻¹) ²
Negative control ³	-	18 ± 1.0	-
CS ⁴	--	10 ± 0.6	25
CS- <i>g</i> -poly(AA) ⁴	--	3.0 ± 0.4	1.56
CS- <i>g</i> -poly(HEMA) ⁴	--	6.6 ± 3.5	> 25
CS- <i>g</i> -poly(AA- <i>co</i> -HEMA) ⁴	--	1.2 ± 0.5	12.5
CS- <i>g</i> -poly(AA- <i>co</i> -HEMA) ⁴	5	1.1 ± 0.4	12.5
CS- <i>g</i> -poly(AA- <i>co</i> -HEMA) ⁴	7	5.1 ± 0.6	12.5
CS- <i>g</i> -poly(AA- <i>co</i> -HEMA) ⁴	10	4.6 ± 0.4	12.5
CS- <i>g</i> -poly(AA- <i>co</i> -HEMA) ⁴	15	4.9 ± 4.9	12.5

¹CFU ml⁻¹ is shown as the mean ± 1 SD and is derived from 3 repeats. Means within the column are significantly different (p<0.05).

²MIC are shown as mg ml⁻¹ for a 2% (v v⁻¹) seeding of a late log phase culture of *S. aureus* in MHB media and evaluated at late stationary phase (24 hr of culture).

³Negative control contains only the MHB medium and *S. aureus*.

⁴All hydrogel samples were co-cultured with *S. aureus* in MHB media at 250 mg ml⁻¹.

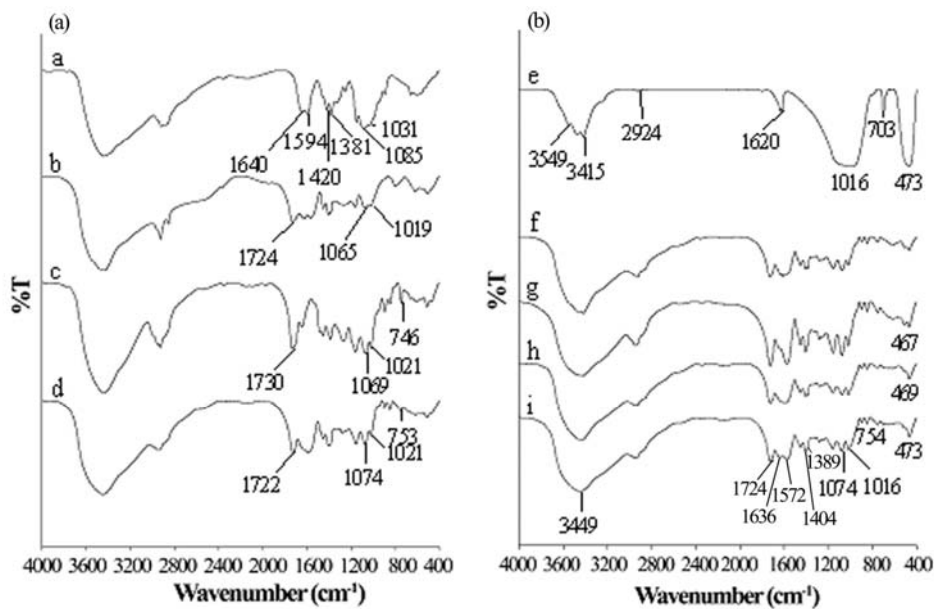


Figure 1

Representative FT-IR spectra of (A): (a) CS, and (b) CS-g-poly(AA), (c) CS-g-poly(HEMA) and (d) CS-g-poly(AA-co-HEMA) hydrogels, the last being synthesized with an AA: HEMA, N-MBA: CH, APS: CH and TEMED: CH molar ratios of 1, 0.11, 0.07 and 0.58, respectively; (B): (e) mica, and CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels with mica loadings of: (f) 5%, (g) 7%, (h) 10% and (i) 15% w w-1 based on the monomer.
104x72mm (600 x 600 DPI)

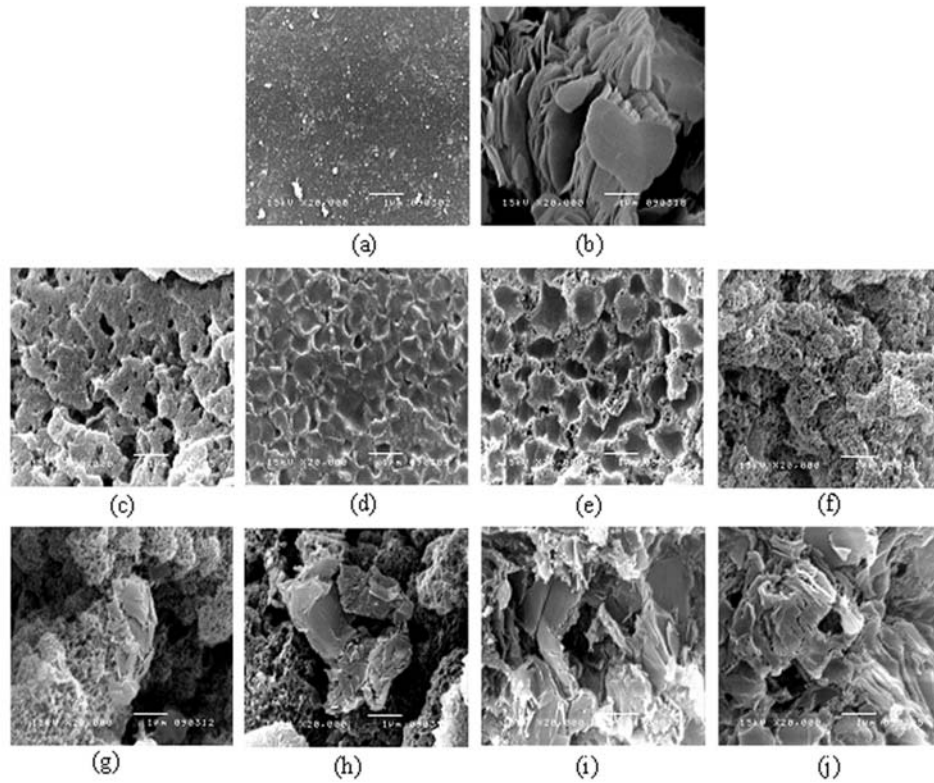


Figure 2

Representative SEM micrographs of (a) CS, (b) mica, (c) CS-g-poly(AA), (d) CS-g-poly(HEMA), (e, f) CS-g-poly(AA-co-HEMA), and CS-g-poly(AA-co-HEMA)/mica composite hydrogels synthesized with mica loading levels of (g) 5%, (h) 7%, (i) 10% and (j) 15% w w-1 of the monomer. 158x140mm (400 x 400 DPI)

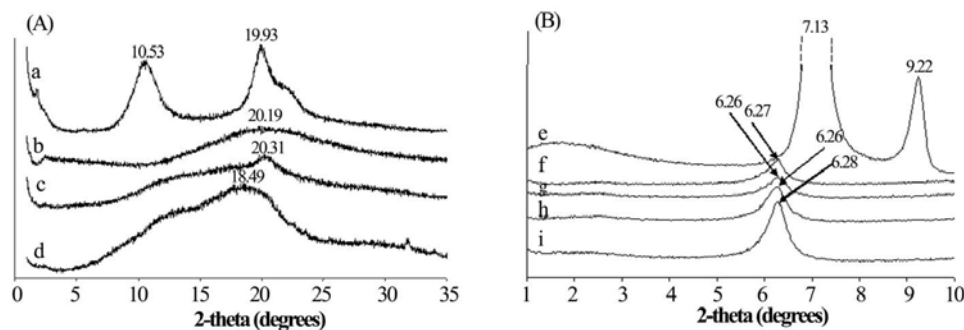


Figure 3

Representative XRD patterns of hydrogels synthesized from (A): (a) CS, (b) CS-g-poly(AA) synthesized with 1 g CS, 54 mmol AA, and N-MBA, APS and TEMED at 10%, 10% and 40% w w-1 of CS, respectively, (c) CS-g-poly(AA-co-HEMA) and (d) CS-g-poly(HEMA); (B): (e) mica, and CS-g-poly(AA-co-HEMA)/mica nanocomposite hydrogels synthesized with mica loadings of (f) 5%, (g) 7%, (h) 10% and (i) 15% w w-1 based on the monomer, 1 g CS, an AA: HEMA molar ratio of 1:1, and N-MBA, APS and TEMED at 10%, 10% and 40% w w-1 of CS, respectively.

60x24mm (600 x 600 DPI)

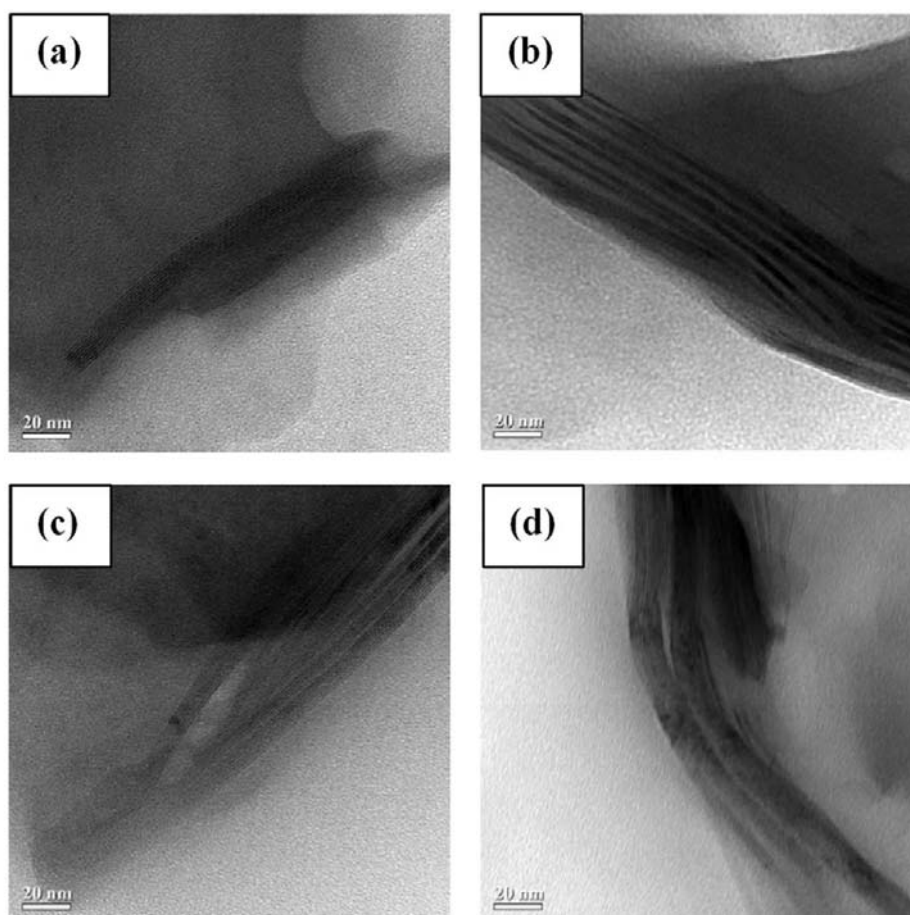


Figure 4

Representative TEM micrographs of CS-g-poly(AA-co-HEMA)/mica composite hydrogels synthesized with mica loadings of (a) 5%, (b) 7%, (c) 10% and (d) 15% w w-1 based on the monomer.
104x115mm (300 x 300 DPI)

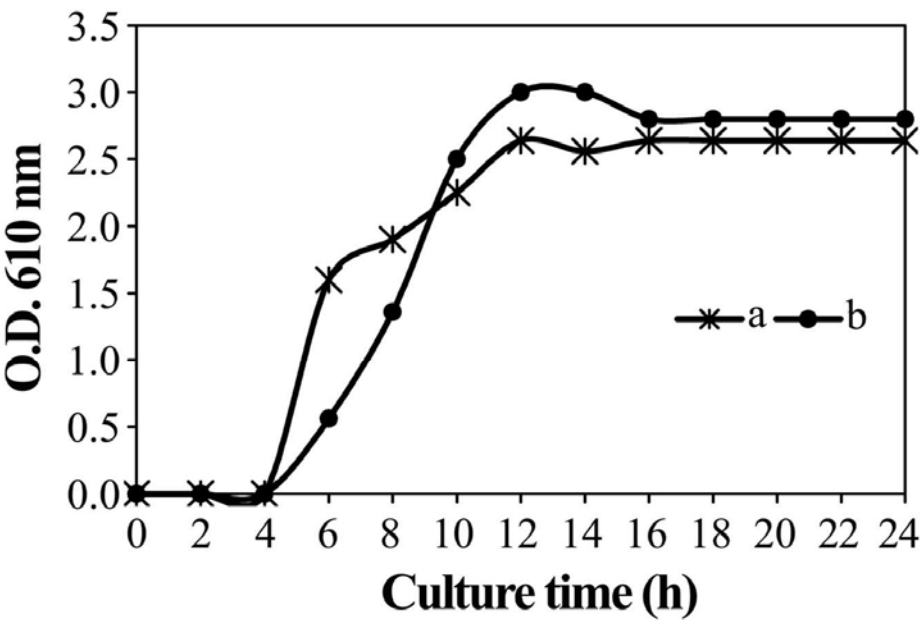


Figure 5

The optical density at 610 nm of *S. aureus* cultured in HMB media at various culture times with (a) the negative control, or 250 mg ml⁻¹ of (b) CS-g-poly(AA-co-HEMA) hydrogel prepared with 1 g CS, a AA: HEMA molar ratio of 1:1, and N-MBA, APS and TEMED at 10%, 10% and 40% w w⁻¹ of CS, respectively, with polymerization performed at 250 pm and 60 °C for 60 min.
59x45mm (600 x 600 DPI)

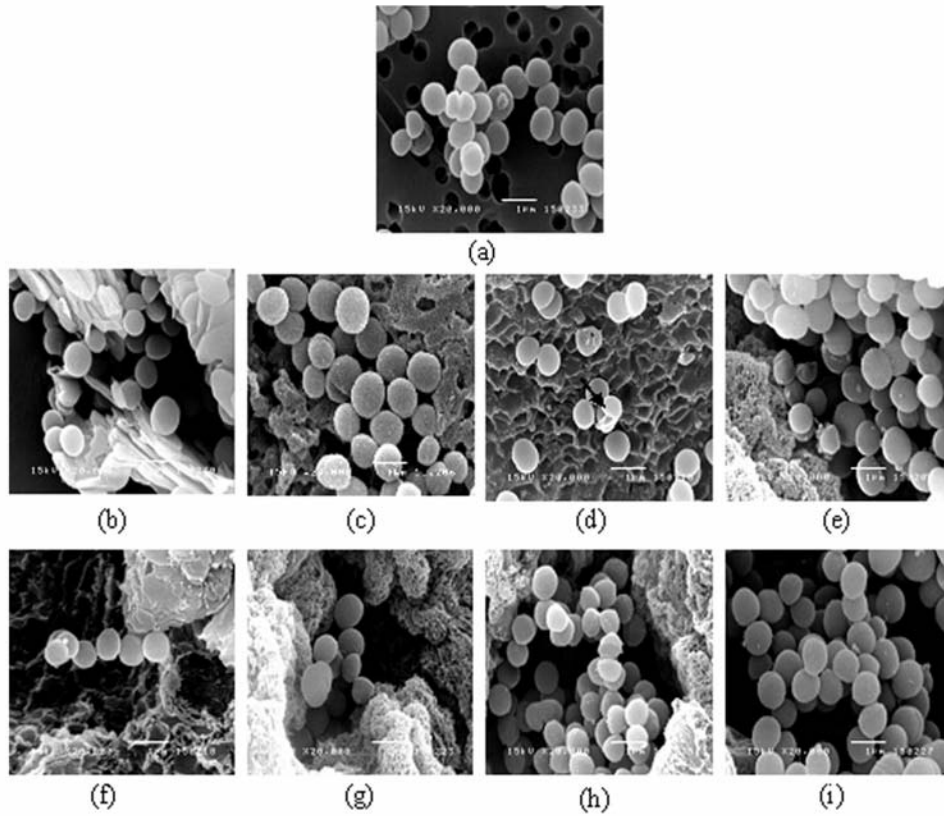


Figure 6

Representative SEM micrographs of *S. aureus* after co-culture for 24 h with (a) negative control, (b) mica, (c) CS-g-poly(AA), (d) CS-g-poly(HEMA), (e) CS-g-poly(AA-co-PHEMA) and CS-g-poly(AA-co-PHEMA)/mica nanocomposites with mica loadings of: (f) 5%, (g) 7%, (h) 10% and (i) 15% w w-1 based on the monomer. The arrow in (d) highlights a damaged cell.

152x142mm (600 x 600 DPI)

KAPOK I: CHARACTERISTICS OF KAPOK FIBER AS A POTENTIAL PULP SOURCE FOR PAPERMAKING

Somporn Chaiarrekij,^{*} Apiporn Apirakchaiskul, Kuntinee Suvarnakich, and Suda Kiatkamjornwong

The potential use of kapok fiber for pulping and papermaking has been investigated. The kapok fibers were cooked using the optimal dosage of sodium hydroxide determined from the experiments. Then, the pulp was refined twice using a disc refiner with a disc gap of 1/100 inch and this kapok pulp was mixed with commercial hardwood pulp and/or softwood pulp at different blend ratios to make papers. Addition of the kapok pulp to the mixed pulps improved the tensile and burst strengths of the sheets but decreased the tear resistance and elongation. Brightness and opacity of the sheets were also dropped with the addition of kapok pulp to the mixed pulps. Water repellency of the sheets prepared from the kapok pulp mixed with the commercial pulps was also improved. These results indicate that kapok fiber can be a quality pulp source for papermaking, especially for packaging paper.

Keywords: Kapok fiber; Properties; Raw material; Papermaking

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INTRODUCTION

The kapok tree, *Ceiba pentandra* (L.) Gaertn. (Malvales: Malvaceae), formerly Bombacaceae family, is cultivated widely in Southeast Asia, as well as other parts of East Asia and Africa. The kapok fiber is an agricultural product obtained from the fruits of the kapok tree (Tang et al. 2008; Qiuling and Lin 2009). Kapok fibers have a hollow structure with a thin fiber wall and large lumen (Hong et al. 2005; Lim and Huang 2007). The diameter including the fiber wall is $16.5 \pm 2.4 \mu\text{m}$. The lumen diameter is $14.5 \pm 2.4 \mu\text{m}$ and the fiber length is $25 \pm 5 \mu\text{m}$ (Huang and Lim 2006). Kapok fibers are fluffy, light-weight and too inelastic to be spun, and so are good for stuffing beds, pillows and cushions. Chemical compositions of kapok fiber were differently reported by two groups of researchers. The first one mentioned that kapok fiber is chemically composed of 64% cellulose, 13% lignin and 23% pentosan on a weight basis (Kobayashi et al. 1977) while the other stated that it comprises of 35% cellulose, 21.5% lignin, 22% xylan and 13% of acetyl groups on a weight basis (Hori et al. 2000). Besides all those mentioned chemical compositions, they also contain a waxy cutin on the fiber surface which makes them water repellent and oil absorbent. Thus, they are also useful in oil removal applications (Hori et al. 2000; Khan et al. 2004; Huang and Lim 2006; Rengasamy et al. 2011).

There are few reports on utilizing blended kapok fibers for papermaking, such as, the blend of 75:25 (w/w) kapok fibers: bamboo pulp was treated with detergent and

sodium hydroxide before being used to produce paper (Malab et al. 2001). In this case, the combination of the two non-wood fibers significantly reduced the volume of the bamboo pulp, and the amounts of chemical reagents and energy consumption, without adversely affecting the paper strength properties. Another study has indicated that kapok fibers could potentially be pulped to produce paper (Chaiarrekij et al. 2008), but this claim has not been established and any products have not been further characterized. However, it has been observed that paper sheets made from kapok pulp seemed to demonstrate superior water resistant behavior even without any sizing agents being added.

Consequently, the optimum set of pulping conditions for the kapok fibers to give the best strength properties was investigated. This optimal set of pulping conditions was then employed to produce kapok pulp. The influence of kapok pulp on paper properties when the kapok pulp was mixed with commercial hardwood pulp and softwood pulp was also determined. The hypothesis being tested was that kapok pulp might increase the strength properties of the hybrid paper prepared from mixtures of kapok pulp with conventional commercial softwood pulp and/or hardwood pulp. It was thought that as well as providing better strength, paper made of kapok pulp might possess superior water resistance, which is generally required for a packaging paper.

EXPERIMENTAL

Materials

Before pulping, the kapok fibers (sourced from the north eastern part of Thailand) were washed, cleaned, and immersed in water for three weeks prior to manually squeezing out the excess water and cutting to 3-5 cm in length. The moisture content was determined using the moisture balance (FD-600, Kett Electric Laboratory, Japan). The commercial softwood (SW) pulp from Pine and hardwood (HW) pulp from Eucalyptus were obtained from the Crofton Pulp & Paper Mill, Canada and the Phoenix Pulp & Paper Public Co., Ltd, Thailand, respectively.

Methods

The experiment was divided into 2 parts. The first part was about determination of optimal pulping condition for kapok fiber, while the second part was about examining the effects of kapok pulp addition to commercial softwood and hardwood pulps on the properties of the resultant pulp and paper.

Determination of optimal pulping condition for kapok fiber

Kapok fibers were separately pulped using 10, 15, 20, and 25% (w/w) NaOH, based on the oven-dried pulp weight. The liquor-to-wood ratio was 17:1. The pulping was carried out in an autoclave digester, (UEC-2017A, Universal Engineering, India) with an initial digesting temperature at 40 °C. The pulp matrix was then gradually heated to 120 °C over 30 min. Pulping was proceeded at this temperature for another 120 min. The pulp was then extensively washed with tap water to remove the alkalinity from the pulp by controlling with a pH meter (Hanna HI 98128, Hanna Instrument, U.S.A.).

Kapok pulps were refined twice through a disc refiner, using a 1/100 inch disc gap (Andritz Sprout, U.S.A.). The degree of refining was determined following the Canadian Standard Freeness (CSF) standard, using a freeness tester (CF/A, Regmed, Brazil) according to TAPPI Standard Method T227 om-04. Kapok pulps were disintegrated in a standard disintegrator (Formax T-100, Adirondack, U.S.A.) for 100,000 revolutions in hot water (80 °C) to remove latency. The average length of 5,000 fibers per sample was automatically determined using a fiber quality analyzer (FQA, Optest, Canada) according to the TAPPI Standard Method T271 om-98 using the following equations.

The arithmetic average length of individual fibers (L) is calculated from equation (1):

$$L = \frac{\sum n_i l_i}{\sum n_i} \quad (1)$$

The length-weighted average length of the fibers (L_l) is calculated from equation (2):

$$L_l = \frac{\sum n_i l_i^2}{\sum n_i l_i} \quad (2)$$

The weight-weighted average length of the fibers (L_w) is calculated from equation (3):

$$L_w = \frac{\sum n_i l_i^3}{\sum n_i l_i^2} \quad (3)$$

where the number of fibers (n_i) is the number of fibers in each class of length (l_i).

The chemical composition of each pulp was determined using TAPPI Standard Method T222 om-98 for lignin determination, TAPPI Standard Method T203 cm-99 for alpha cellulose (α -cellulose) determination, and Browning's method for holocellulose determination (Browning 1963). The amount of hemicellulose was calculated from the difference between the holocellulose content and the alpha cellulose content.

Kapok pulp was also made into 60 g/m² handsheets on a Rapid-Köthen sheet former (RK-2A KWT, PTI, Austria) according to the ISO Standard Method 5269-2. The brightness and opacity of the handsheets were measured using an optical tester (Color Touch PC, Technidyne, U.S.A.), based on ISO Standard Methods 2470 and 2471, respectively. The tensile, burst, tear, and zero-span tensile strengths were measured using a tensile strength tester (Strograph E-S, Toyo Seiki, Japan), a burst strength tester

(SE002P, Lorentzen & Wettre, Sweden), a tear strength tester (Protear, Thwing-Albert, U.S.A.), and a modified tensile strength tester (Pendulum Tensile Strength Tester with special clamps adjustable to zero, Toyo Seiki, Japan), according to TAPPI Standard Method T494 om-01, T403 om-02, T414 om-04 and T231 cm-96, respectively. The contact angle of water in contact with surface of handsheet was automatically measured using the Pocket Goniometer PG-3 (Fibro System, Sweden) by capturing and processing the water droplet images. The dynamic mode was used to measure the rate of change of the contact angle as a function of time. The test was done following TAPPI standard T558 om-06.

The effects of the addition of kapok pulp to commercial softwood pulp and hardwood pulps on the properties of the resultant paper samples

Kapok pulp produced by using the set of optimal conditions previously described was bleached using 3% (w/w) aqueous hydrogen peroxide at a 1.5% consistency and 80 °C for 120 min. The product was disintegrated using a disintegrator (Formax T-100, Adirondack Machine, U.S.A.) for 100,000 revolutions in hot water (80 °C) to remove latency. Each commercial pulp was beaten in a valley beater (UEC-2018A, Universal Engineering, India) in which 360 g of the oven-dried pulp in 23 L of water was accommodated, according to TAPPI Standard method T200 sp-01. The softwood pulp and hardwood pulp were separately beaten to meet the Canadian Standard Freeness of 350 mL and 300 mL, respectively. Then, the bleached kapok pulp was mixed with the commercial beaten hardwood pulp, softwood pulp and mixed pulp (25:75 (w/w) softwood: hardwood) to make handsheets. These procedures of papermaking and paper characterization were repeated in the manner as described.

Statistical analysis

Parametric data were analyzed using the analysis of variance (ANOVA). In all cases, a p-value smaller than or equal to 0.05 was accepted as being a statistically significant difference. For all of the experiments, three replicates were performed.

RESULTS AND DISCUSSION

Determination of Optimal Pulping Condition

Chemical composition of kapok pulp

The effects of the sodium hydroxide solution on the chemical composition of the kapok pulp are summarized in Table 1. Analysis of the chemical composition of the pulps showed that an increased amount of sodium hydroxide in cooking liquor resulted in lower percentages of lignin, higher percentages of holocellulose and alpha cellulose, with lower percentages of hemicellulose. It should be noted that holocellulose is composed of alpha cellulose and hemicellulose. This finding was to be expected, since sodium hydroxide solution dissolves lignin. Carbohydrates, especially hemicellulose, can also be easily destroyed by sodium hydroxide, leaving a higher ratio of alpha cellulose behind. Since all of the P-values of the chemical compositions were lower than 0.05, the effects of sodium

hydroxide solution on kapok pulp chemical compositions were thus statistically significant.

Table 1. Effects of Sodium Hydroxide Concentration on the Chemical Composition of the Kapok Pulp

Pulp Chemical Composition	NaOH Dosage (% w/w)				P-value
	10	15	20	25	
Lignin (%)	19.20	18.27	17.57	17.53	0.0109*
Holocellulose (%)	76.12	76.53	78.49	78.50	0.0005*
Alpha cellulose (%)	65.63	69.87	69.65	69.96	0.0002*
Hemicellulose (%)	10.49	6.66	8.84	8.54	0.0007*

* Statistically significant since P-value is lower than 0.05

Kapok pulp and fiber characteristics

The effects of sodium hydroxide treatment level on pulp yield, freeness and the average fiber length, characterized in terms of arithmetic, length-weighted, and weight-weighted criteria are summarized in Table 2, along with the percentage of fines.

Table 2. Pulp and Fiber Characteristics of the Kapok Pulp after Soda Pulping

Pulp and Fiber Properties	NaOH Dosage (% w/w)			
	10	15	20	25
Pulp yield (%)	62.40	51.25	44.69	40.46
Freeness (CSF, mL)	401	362	296	277
Average fiber length-Arithmetic (mm)	0.91	0.91	0.83	0.81
Average fiber length-Length weighted (mm)	1.51	1.49	1.32	1.28
Average fiber length-Weight weighted (mm)	2.26	2.17	1.93	1.83
Fines (%)	21.5	21.6	22.7	23.7

The results in Table 2 indicate that high sodium hydroxide concentration could also result in a lower pulp yield because of the removal of lignin and extractives. Also, high sodium hydroxide dosage could lead to carbohydrate degradation, especially with the hemicellulose, due to a peeling reaction. This might also contribute to the lower pulp yield.

Table 2 shows that increasing the concentration of sodium hydroxide significantly decreased the degree of the pulp freeness. Thus, there was a decrease in the pulp's drainage capacity. This was probably due to an increasing amount of fines which would have a significantly greater surface area than fibers for water absorption. Also relevant are the removal of hydrophobic lignin, the dissolution of hemicellulose and possibly the saponification of the cutin wax by the alkali with "solvation" of the cellulose. The latter two possibilities were supported by the results from contact angle studies in which the contact angle with water decreased when the amount of alkali was increased (Fig. 1).

Figure 1 also shows that the handsheets in the dry state (at a zero contact time) had the greater water contact angles. However, after contact with the water, the contact angles decreased due to wetting, absorption, penetration, and spreading of the water under the influence of the cellulosic hydrophilic interactions.

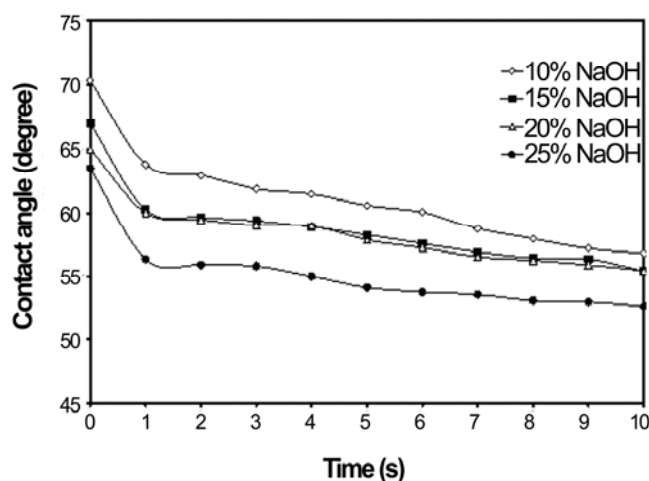


Fig. 1. The effects of different sodium hydroxide concentrations on water contact angles of the kapok fiber sheets.

It should be noted that weight weighted average fiber length is one of the average fiber length values, but it emphasizes more on the content of long fibers as compared to the length weighted fiber length and the arithmetic average fiber length, respectively. When the amount of sodium hydroxide increased, the average fiber length significantly decreased, whilst the fines content was increased (Table 2). This was likely to have occurred because the fibers were damaged by a peeling reaction under the strong alkali conditions leading to carbohydrate degradation (Sjöström 1993). Thus, the greater the amount of carbohydrate that was degraded by the alkali, the lower would be the mean fiber length and the greater would be the amount of fiber fines.

Kapok handsheet properties

The effects of the sodium hydroxide solution on the kapok handsheet properties are summarized in Table 3.

Table 3. Effects of Sodium Hydroxide Concentration on the Kapok Handsheet Properties

Pulp and Paper Properties	NaOH Dosage (% w/w)				P-value
	10	15	20	25	
Apparent density (g/cm ³)	0.68	0.80	0.82	0.88	0.1264
ISO opacity (%)	97.9	97.5	97.4	97.0	0.0235*
ISO brightness (%)	21.3	19.1	18.6	18.1	0.1917
Tensile index (N m/g)	80.3	100.4	111.5	101.3	0.0002*
Elongation (%)	1.75	2.01	2.10	2.05	0.0335*
Burst index (kPa m ² /g)	4.44	5.73	5.96	5.98	0.0348*
Tear index (mN m ² /g)	1.78	1.68	1.60	1.59	0.4005
Zero span tensile index (N m/g)	59.5	58.2	56.7	55.9	0.9801

* Statistically significant since P-value is lower than 0.05

Increasing the amount of sodium hydroxide solution led to an increase in the apparent density of the sheets which was defined as handsheet grammage divided by

handsheet thickness, but a lower opacity (Table 3). Such effects are likely to be due to the role of sodium hydroxide solution in breaking up lignin molecules within the fiber cell wall. As the hydrophobic lignin was removed, the fiber surface became more hydrophilic, encouraging fiber swelling and conformability. This would result in a more compacted sheet that in turn, which could lead to fewer air-to-fiber interfaces and a lower opacity. In addition, the increasing content of fines and the greater amount of fiber contact areas would help to improve fiber bonding, leading to denser sheets, as indicated by the higher apparent density. Shorter fibers and a greater amount of fines also provided a basis for more effective filling of existing voids, which might also have contributed to lower opacity of the sheet. However, the effect of sodium hydroxide solutions on the apparent density appeared in this case to be statistically insignificant while its effect on opacity was statistically significant.

Pulp brightness would be expected to increase when greater amounts of sodium hydroxide solution were used, since more lignin would be removed. However, the observed trend was the opposite of this expectation, as indicated by the statistical evaluation (Table 3). However, greater concentrations of sodium hydroxide solution might reduce pulp brightness through an alkali darkening reaction, resulting in structural changes of the chromophoric groups in the residual lignin. These groups could also absorb light and thus cause the brightness of the pulp to decrease. An additional plausible explanation might be that the sodium hydroxide solution mediated the removal of lignin and caused greater sheet compactness with a resultant reduced level of light scattering of the sheet, whilst the greater amount of fines might also lead to sheet compactness.

The tensile index defines the tensile strength of paper. It is the tensile strength divided by the grammage. The burst index defines the burst strength of the paper and is equal to the burst strength divided by the grammage. Fiber bonding is highly likely to affect the tensile strength and burst strength. Increasing sodium hydroxide dosage provided higher tensile and burst strengths due to increasing fiber bonding, since lignin was removed and fibers became more conformable (Table 3). Increased fiber bonding caused by the greater sodium hydroxide dosage also led to the higher elongation. However, it should be noticed that using too high dosage of sodium hydroxide was detrimental to tensile and burst strengths as a result of carbohydrate degradation by peeling reaction at a strong alkali condition. The effects of the sodium hydroxide concentration in cooking liquor on the tensile index, the elongation and the burst index were statistically significant.

The tear index, the tear resistance value divided by the grammage, defines the tear resistance of the paper. Tear resistance is affected by many factors, such as the intrinsic fiber strength, the fiber length, and fiber bonding. The most important factor for well-bonded sheets is the fiber strength. The zero span tensile index is defined by the zero-span tensile strength divided by the grammage. Generally, zero span tensile index is a function of both the intrinsic fiber strength and the fiber bonding; however, the intrinsic fiber strength is a major contributing factor. The tear index decreased when the sodium hydroxide concentration increased (Table 3). The same observation was found for the zero span tensile index. This effect could be caused by the peeling reaction that destroys endwise, the chains of cellulose and hemicellulose and by the alkaline hydrolysis that leads to a drop in the degree of polymerization of the fibers (Shatalov and Pereira 2005).

The decrease in tear resistance might also be caused by the shorter fiber length and the greater amount of fines that are created. However, the effects of sodium hydroxide dosage on the tear and zero span tensile index values were so small that they were statistically insignificant.

Overall, the optimum condition for kapok fiber pulping was that of the cooking liquor containing 20% (w/w) of sodium hydroxide, which gave the maximum tensile properties as discussed. Thus, kapok pulp that was prepared using a 20% (w/w) sodium hydroxide dosage was used in subsequent experiments. However, the pulp was bleached before being mixed with commercial pulps.

Effects of Kapok Pulp Addition to Commercial Softwood and Hardwood Pulps on the Properties of the Resultant Pulp and Paper

Fiber length distributions of kapok, softwood and hardwood pulps

The fiber length distributions of the kapok pulp that was produced using the cooking liquor of 20% (w/w) sodium hydroxide and the commercial softwood pulp and hardwood pulp are shown in Fig. 2.

The kapok pulp had an intermediate amount of short fibers that was less than that of the hardwood pulps. The softwood pulp gave the lowest short fiber content. The kapok pulp was also intermediate in the amount of both medium and long fibers. These were lower than that from the hardwood pulp. The softwood pulp contained the greater amount of long fibers.

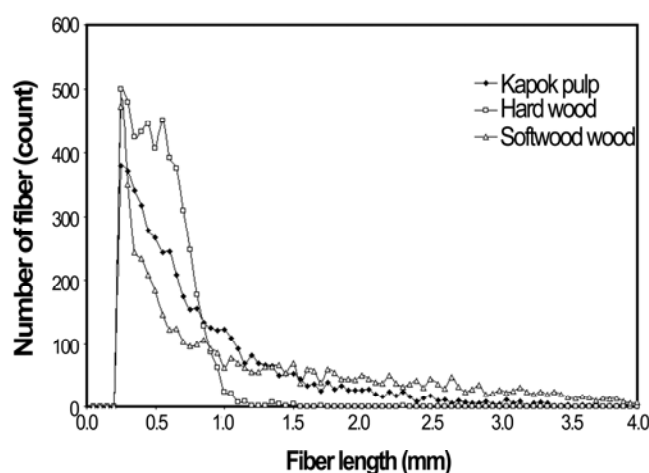


Fig. 2. Fiber length distributions of the kapok, hardwood and softwood pulps.

Fiber length, fines content and zero span tensile index of kapok, softwood and hardwood pulps

Fiber length, fines content and zero span tensile index of kapok, softwood and hardwood pulps are compared and illustrated in Table 4.

Table 4. Fiber Length, Fines Content and Zero Span Tensile Index of Kapok and the Commercial Pulp

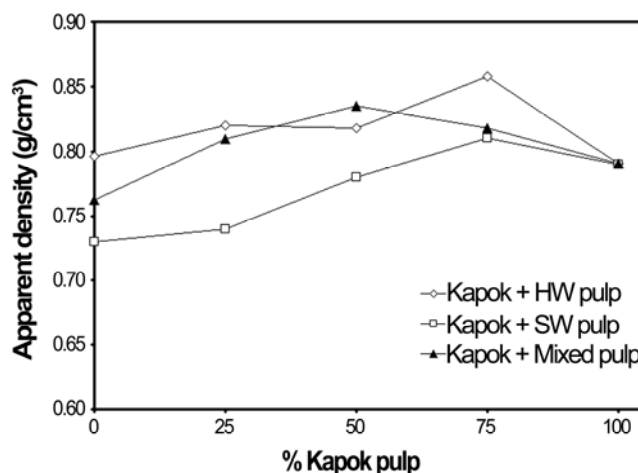
Pulp Properties	Pulp Types		
	Kapok	Softwood (SW)	Hardwood (HW)
Average fiber length-Arithmetic (mm)	0.83	1.25	0.50
Fines (%)	22.7	43.2	34.0
Zero span tensile index (N m/g)	56.7	82.6	44.2

After being refined to the freeness of 300 mL as shown in Table 4, the average fiber length of the kapok pulp (0.834 mm) was between those of the softwood pulp (1.253 mm) and hardwood pulp (0.496 mm). The kapok pulp also had the lowest level of fines, followed by the hardwood pulp and softwood pulp, respectively.

As shown in Table 4, the sheets made from the softwood and hardwood pulps had the greatest and least zero-span tensile index at 82.6 and 44.2 N m/g, respectively. That made from kapok pulp was 56.7 N m/g. Since kapok fiber was brittle, this led to the lower zero span tensile index of kapok pulp.

Apparent density

The apparent densities of the sheets that were prepared from different ratios of the kapok pulp to the commercial softwood (SW), hardwood (HW) and softwood-hardwood mixed pulps, are shown in Fig. 3.

**Fig. 3.** Apparent density of the sheets made from kapok pulp mixed with the commercial hardwood (HW) pulp and softwood (SW) pulp at the indicated percentages.

The sheets made from the hardwood pulp had a greater apparent density than those containing either the softwood pulp or the kapok pulp. That is because the shorter fibers of the hardwood pulp fill the voids and pores in the sheet structure, making the sheets more compact. The inclusion of kapok pulp increased the apparent density of the sheets in all of the pulp mixtures. This could be because the kapok fibers have very thin cell wall, being easily collapsed, making more compact sheets. However, sheets derived

from the pure kapok pulp were not greater in apparent density, as seen by the fact that the kapok fibers become easily entangled. Thus, pure kapok sheets gave poor sheet formation and an irregular sheet surface.

Tensile index and burst index

The sheets made from kapok pulp gave the highest tensile index (Fig. 4). This could be because the kapok fibers were long and the lumens were easily collapsed when forming paper, producing highly bonded regions and denser sheets with a resulting superior sheet tensile index.

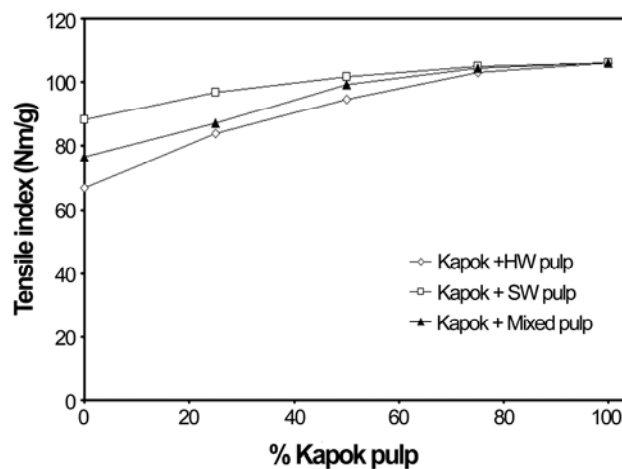


Fig. 4. Tensile index of the sheets made from kapok pulp mixed with the commercial hardwood (HW) pulp and softwood (SW) pulp at the indicated percentages.

The sheets made from the hardwood pulp produced the lowest tensile index. Those made from a 75:25 (w/w) blend of the hardwood: softwood pulps had a medium tensile index. Those made from the pure softwood pulp gave the greatest tensile index. With respect to kapok-commercial pulp mixtures, as the ratio of kapok pulp increased, the tensile index also increased (Fig. 4). However, the results were more prominent when the kapok pulp was mixed with the hardwood pulp than with the softwood pulp.

The sheets derived from the pure kapok pulp gave the highest burst index, the values being somewhat similar to those of the sheets made from the softwood pulp (Fig. 5). This is because both kapok fiber and the softwood fiber are long and have extensive contact areas that are available for fiber bonding. The sheets made from the hardwood pulp had the lowest burst index. This is because the hardwood pulp contained a greater amount of short fibers, resulting in poor bonding. As the ratio of the kapok pulp in mixtures with the hardwood pulp or the 75:25 ratios (w/w) of hardwood pulp: softwood pulp increased, the burst indexes of the resultant paper significantly increased, because fiber bonding was greatly improved.

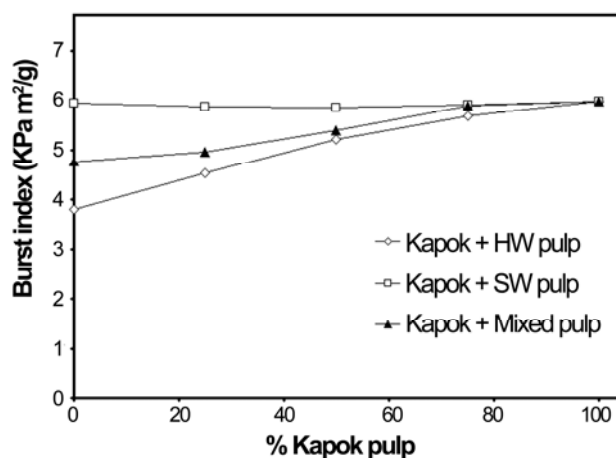


Fig. 5. Burst index of the sheets made from kapok pulp mixed with the commercial hardwood (HW) pulp and softwood (SW) pulp at the indicated percentages.

Tear index and elongation

One of the key characteristics of kapok fibers is their brittleness. Thus, the sheets made from the kapok pulp had the lowest tear index relative to those made from the commercial softwood pulp, the hardwood pulp, and the softwood-hardwood mixed pulp. For kapok-commercial pulp mixtures at different ratios, it was clearly observed that as the ratio of kapok pulp increased, the tear index significantly decreased (Fig. 6).

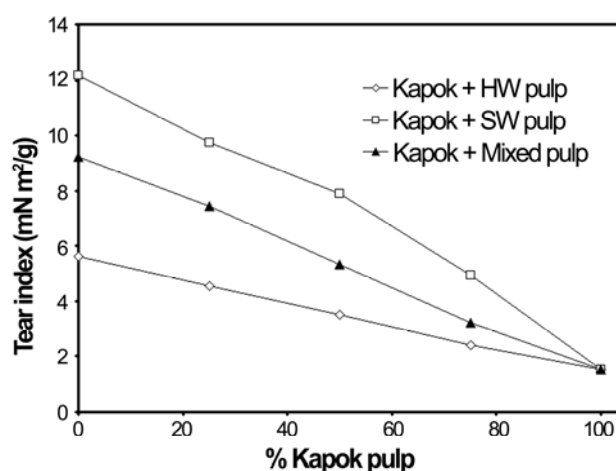


Fig. 6. Tear index of the sheets made from kapok pulp mixed with the commercial hardwood (HW) pulp and softwood (SW) pulp at the indicated percentages.

The elongation of the sheets that were prepared from different ratios of the kapok pulp is compared in Fig. 7. Sheets made from kapok pulp had the lowest elongation. This is because the key characteristics of kapok fibers are their brittleness and inelasticity, which might reduce the elongation of the sheets when a load was applied. Thus, as the

ratio of the kapok pulp was increased, the elongation significantly decreased in all pulp mixtures.

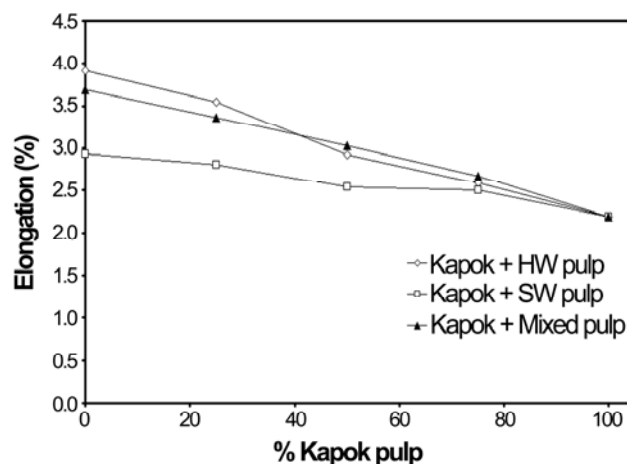


Fig. 7. Elongation of the sheets made from kapok pulp mixed with the commercial hardwood (HW) pulp and softwood (SW) pulp at the indicated percentages.

Water contact angle

The water contact angles of the sheets made from kapok pulp mixed with the commercial softwood pulp and hardwood pulp are shown in Fig. 8. As seen in Fig. 8, K 100 represented handsheets made from 100% kapok pulp. HW100 indicated handsheets made from 100% hardwood pulp while SW100 illustrated handsheets made from 100% softwood pulp. M100 designated handsheets made from mixed pulp (25:75 (w/w) softwood pulp: hardwood pulp). Handsheets prepared from kapok pulp mixed with hardwood, softwood and mixed pulps with different percentages characterized by K: HW, K: SW and K: M with the percentage indicated in parenthesis.

The results indicated that as the time of contact increased, the water contact angles decreased because of absorption and penetration of the water droplets into the sheet. Those made from 100% kapok pulp gave the greatest values for the water contact angle. This effect was due to the unique characteristics of the kapok fibers having a wax coating on the surface. Although the kapok fibers were pulped, some of the wax was still present. When mixed with the commercial pulps (softwood, hardwood and mixed pulps), as the ratio (w/w) of the kapok pulps increased, the water contact angle values increased.

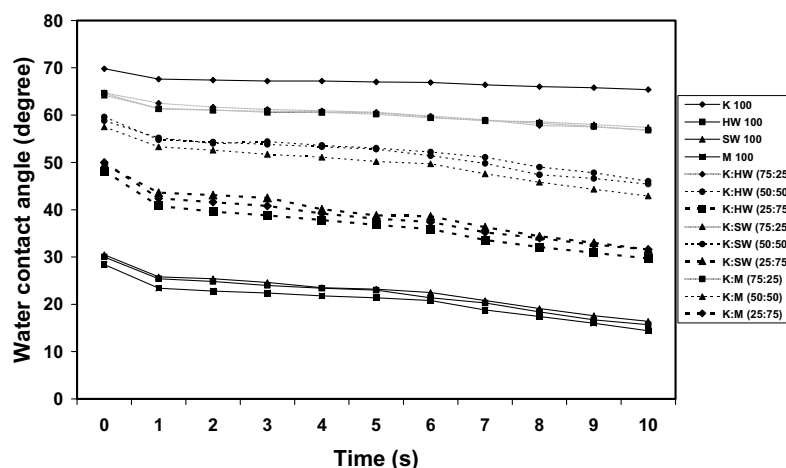


Fig. 8. Water contact angles of the sheets from the kapok (K) pulp mixed with the commercial hardwood (HW) pulp, softwood (SW) pulp and mixed (M) pulp at the indicated percentages.

CONCLUSIONS

1. When kapok pulp was mixed with the commercial softwood pulp and/or hardwood pulp, the tensile and burst strengths of the blended sheets increased but with the lower tear resistance and elongation due to the excellent tensile strength and brittleness of kapok pulp.
2. Brightness and opacity of the blended sheets decreased when higher amounts of kapok pulp were added in the pulp mixture due to the yellowness of the kapok pulp.
3. Handsheets containing kapok pulp gave greater water contact angles due to the hydrophobic nature of kapok fibers.
4. Thus, kapok pulp can potentially be mixed with the commercial softwood pulp and/or hardwood pulp to produce commercial packaging paper when the strength and light weight characteristics are required.

ACKNOWLEDGMENTS

This research was financially supported by a Research Team Aid Grant from the Thailand Research Fund and Commission of Higher Education under the contract number RTA5080004. The authors would like to thank the Department of Imaging and Printing Technology of Chulalongkorn University, and the Product and Technology Development Center of Siam Cement Group Paper Company Limited for providing relevant research facilities.

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Article resubmitted: July 31, 2011;

Elsevier Editorial System(tm) for Journal of Chromatography A
Manuscript Draft

Manuscript Number:

Title: Comparative study of the mesostructure of natural and synthetic polyisoprene by SEC-MALS and AF4-MALS

Article Type: Full Length Article

Keywords: Field flow fractionation; size exclusion chromatography; polyisoprene; multi angle light scattering; rubber

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Montpellier, 05th July 2011

Cover letter

Dear Managing Editor,

Please find enclosed the submission of our regular research paper entitled: "*Comparative study of the mesostructure of natural and synthetic polyisoprene by SEC-MALS and AF4-MALS*". In this article, we present the first original results about natural and synthetic rubber characterization by asymmetrical flow field flow fractionation (AF4). Mesostructure evaluation by AF4 is compared with that obtained with size exclusion chromatography.

Up to now, there is a real lack of information concerning natural rubber mesostructure and more precisely about the presence and the nature of microaggregates (gel content) among the linear polyisoprene chains. AF4 allows assessing simultaneously both the distribution of polyisoprene chains and gel content. Results obtained allow to validate the AF4 fractionation (compared to SEC results) and to consider the gel part of polyisoprene samples.

For these reasons, we think that our article matches with the topic of your journal and is appropriate for publication in journal of chromatography A.

Sincerely yours,

The authors: Stéphane Dubascoux, Chalao Thepchalerm, Eric Dubreucq, Suwaluk Wisunthorn, Laurent Vaysse, Suda Kiatkamjornwong, Charoen Nakason, Frederic Bonfils

Comparative study of the mesostructure of natural and synthetic polyisoprene by SEC-MALS and AF4-MALS.

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Abstract

This paper presents results from the first analyses of the mesostructure of natural rubber (NR) by asymmetrical flow field flow fractionation (AF4). The results are compared with those obtained by size exclusion chromatography (SEC) in terms of average molar masses, radius of gyration and insoluble part (or gel quantity). Comparable results were obtained for the sample not containing gel. Conversely, for samples with gel, significant differences were found due to the presence of microaggregates. In fact, contrary to SEC, AF4 fractionation enables partial fractionation of polyisoprene chains and microaggregates in a single run without preliminary treatment. The results presented here also highlight the special structure (very compact spheres) of microaggregates in NR compared to chemical crosslinked microaggregates in synthetic polyisoprene. The advantages and drawbacks of both techniques for analysing NR samples are also discussed.

Introduction

For natural rubber (NR), as for numerous biopolymers, it is rather simplistic to speak about macromolecular structure. Indeed, biopolymers often exhibit a complex associative structure, a mixture of macromolecular chains, microaggregates and macroaggregates [1,2]. Because of that, the term mesoscopic structure or mesostructure, which includes both macromolecular structure and aggregate characterization, is increasingly being used. Although NR mesostructure has been studied for many years, the origins of its unique properties are not yet fully clear. Recently, Kim *et al.* [3,4] revisited the mesostructure of NR. Kim *et al.* [3,4] analysed NR samples with SEC-MALS and showed that the soluble part injected into a SEC system contained very few branched macromolecules, contrary to earlier published studies [5-7]. Kim *et al.* [3,4] showed that the soluble part of NR in solution in tetrahydrofuran (THF) was composed of a mixture of linear chains and assumed compact microaggregates ($R_g \approx 110$ -130 nm). They showed that to more effectively ascertain the mesostructure of NR, as is the case with most polymers, a MALS detector coupled with SEC is required to avoid a misunderstanding of the structure. From a mechanistic point of view, many mechanisms have been proposed to explain the associative structure of NR [8-11]. The most recent proposal is Tanaka's group scheme involving a protein and a phospholipid at each end of the poly(*cis*-1,4-isoprene) chain. These two reactive end chains would appear to be involved in what they called the "naturally occurring network" of NR [12,13].

Today, many tools are available for macromolecule analysis. Of these techniques, size exclusion chromatography (SEC) and field flow fractionation (FFF) are tools of choice to fractionate macromolecules according to their sizes. SEC is beyond doubt the most popular and developed technique for polymer separation. However, a recent study has also highlighted some difficulties for NR separation with SEC coupled with a multi-angle light scattering (MALS) detector because of an abnormal elution phenomenon [3]. This abnormal elution is

55 due to co-elution of microaggregates with short chains. Kim *et al.* [3] showed that the
56 abnormal elution occurring with NR could be overcome by treating the SEC columns with an
57 ionic surfactant. After treatment, it was possible to separate these compact microaggregates
58 from linear random coil polyisoprene chains [3]. However, it is difficult to be sure whether or
59 not all microaggregates elute and the procedure is rather cumbersome. The solution to this
60 SEC limitation for NR sample analysis might be the use of techniques based on field flow
61 fractionation. Recent reviews have demonstrated the potentiality of FFF in various fields,
62 such as polymer science [14], biomolecule characterization [15], nanoparticles [16] or the
63 environment [17]. Moreover, FFF could easily be coupled on-line with different detectors,
64 such as a refractive index detector, an ultra-violet detector, a light scattering or inductively
65 coupled plasma mass spectrometer [18]. Recently, Messaud *et al.* [14] emphasized the fact
66 that field flow fractionation techniques can provide effective simultaneous separation of
67 polymer and microgel. The most popular FFF techniques used for polymer fractionation are
68 thermal flow field flow fractionation (Th-FFF) and Flow Field Flow Fractionation (Fl-FFF or
69 F4) [14]. The main difference between these two sub-techniques is the field used for the
70 fractionation. Fractionation takes place in a channel and the field is a thermal gradient for Th-
71 FFF, while it is a crossflow stream carried through an ultrafiltration membrane in the case of
72 Fl-FFF [19]. Up to now, only Th-FFF has been used for NR characterization [20-23].
73 However, during Th-FFF, macromolecules are separated according to their size and their
74 chemical composition. Thus, heterogeneity in the chemical composition of a given polymer
75 can complicate the determination of macromolecular structure. Asymmetrical Fl-FFF (AsFl-
76 FFF or AF4) is Fl-FFF where the channel is semi-permeable and asymmetric, involving less
77 sample dilution [19]). Natural rubber has been analysed by Th-FFF using either the
78 polyisoprene calibration curve [20,22,23] or a multi-angular light scattering detector (MALS)
79 [21]. Lee and Molnar [23] compared the analysis of natural rubber by Th-FFF and SEC. They

used a calibration curve made with linear standards poly(*cis*-1,4-isoprene). The analysed NR samples (RSS1 type) had a number-average molar mass (M_n) of about 0.8 million $\text{g}\cdot\text{mol}^{-1}$ and a weight-average molar mass (M_w) of about 29 million $\text{g}\cdot\text{mol}^{-1}$, M_w being three times higher than that given by SEC. Lee and Molnar [23] showed that the high molar masses and microgels disappeared after mastication of a NR sample in an extruder. The range of molar masses found was between 10,000 and 10 million $\text{g}\cdot\text{mol}^{-1}$ (non-masticated RSS). Filtration of the solution through filters with 5 μm pores led to a shift in the fractogram towards lower retention times. This phenomenon was observed for non-masticated rubber but not for masticated rubber. This shift to lower molar masses was attributed to the removal and/or degradation of microgels (or ultra-high molar mass species). They quantified the gel rate (as a % of total rubber, w/w) in the NR samples by Th-FFF and found slightly lower values (RSS 13%, masticated RSS 0%) than with their conventional method – i.e. filtration through a 125 μm wire mesh after 24 h in toluene (RSS 15%, masticated RSS 0%). Fulton and Groves [21] analysed two synthetic polyisoprenes (IR305, Natsyn2200), a polybutadiene (Europrene BR40) and a natural rubber (SMR5L) in cyclohexane by Th-FFF-MALS without any filtration. They showed that the IR305 sample was essentially linear with no gel, as confirmed later by Kim *et al.* [3] by SEC-MALS, contrary to the Natsyn2200 sample, which had a 25% gel rate and was branched for molar masses above $5\cdot 10^5 \text{ g}\cdot\text{mol}^{-1}$. While there was no steric effect in the separation process for the two synthetic polyisoprenes, they observed such an effect for the polybutadiene and the NR samples. As a consequence of this phenomenon, very large entities eluted together with the smaller molecules. Bang *et al.* [24] used AF4 to characterize and determine the molar mass distribution of styrene–butadiene rubber.

This paper presents the first study of synthetic and natural polyisoprene fractionation by AF4 coupled with MALS in an organic solvent (tetrahydrofuran). The peak shape is discussed and the results in terms of average molar masses, radius of gyration and gel content are compared

105 with those obtained by SEC-MALS. The comparison of AF4-MALS with SEC-MALS also
106 made it possible to evaluate the discrepancies between the two techniques for analysing NR
107 samples.

108

Material & methods

Samples

In order to compare SEC and AF4 results on synthetic polyisoprene and natural rubber (NR), four samples were chosen for their differences in mesostructure. The two synthetic poly(*cis*-1,4-isoprenes) were Kraton IR 307 (Kraton polymer, Houston, USA) and Nippol 2200 (Zeon Corporation, Louisville, USA). The main difference between these two synthetic polyisoprenes is the gel rate: no gel in the IR307 sample and presence of microgel in Nippol 2200. The two natural rubbers, M160 and M121, were TSR5CV (Technically Specified Rubber with a Constant Viscosity) made from monoclonal *Hevea brasiliensis* latex. The preparation of the samples was described previously [25].

In order to reach a sample concentration of 1 mg.mL⁻¹, 30 ± 5 mg of the samples was dissolved in about 30 mL of HPLC grade tetrahydrofuran (THF, VWR, West Chester, USA) stabilized with 2,6-di-tert-butyl-4-methylphenol – BHT – (Sigma Aldrich, Saint Louis, USA) at 250 mg.L⁻¹. The flasks were precisely weighed (± 0.01 mg) before and after filling with THF to determine the exact concentration of the solutions. The solvent (THF + BHT) was filtered at 0.1 µm before use. Each sample was analysed in triplicate to evaluate measurement repeatability. All solutions were stored in the dark in a water bath at 30°C for 7 days and gently shaken at 30 rpm (rotational agitator REAX 2, Heidolph, Schwabach, Germany) for 1 hour each day for optimum dissolution. Before injection, the macrogel and part of the microgels were removed from all the solutions by filtration at a 1 µm cutoff with Acrodisc glass filters (Pall, Port Washington, USA) to avoid both clogging of the column during SEC analysis and a steric effect (resulting in inverse elution) on AF4 fractionation [21].

Two monodisperse polystyrene standards at 200 and 1460 kDa from Polymer Standard Service (PSS, Mainz, Germany) were used to assess the quality of the separation/fractionation and detector response for each SEC/AF4 injection series.

AF4 experiments

The AF4 system was a Postnova AF2000 MT series (Postnova Analytics GmbH, Landsberg, Germany) equipped with a channel adapted for organic solvents and a 350 μm spacer. The membrane was made of cellulose material treated for compatibility with organic solvents with a cut-off of 5 kDa (Postnova Analytics GmbH, Landsberg, Germany). The temperature setpoint of the AF4 oven containing the channel was 45°C. The detector flow was kept constant at 0.65 mL.min⁻¹. The focusing step consisted of a flow delivered by the injection port of about 0.2 mL.min⁻¹ with a crossflow of 1 mL.min⁻¹ for 6 minutes. Then, a 1 min transition time was applied to avoid a major pressure drop during the switch from the focus step to the elution step. The programme to decrease the linear crossflow during the elution step was as follows:

Table 1

An autosampler (PN 5300 model, Postnova Analytics GmbH) was used to carry out the 100 μL sample injections.

Detection was carried out with a 7-angle multi-angle light scattering detector (PN 3070 model, Postnova Analytics GmbH) in line with a refractive index detector (2414 model, Waters Corporation, Milford, USA). The data gathered were processed with AF2000 software (Postnova Analytics GmbH) after blank subtraction for the DRI signal (used as a concentration detector) and according to the Berry model with 2nd order polynomial formalism. In fact, it has been shown that this formalism is well adapted for entities with a radius up to 50 nm [26]. As the refractive index detector signal changes during transition steps due to the AF4 functioning principle, five blank (mobile phase) injections were carried out in

each injection series. The refractive index detector signal from the blank injections was subtracted for each sample injected.

For one series, each sample triplicate was injected once. Two series were carried out (i.e. a total of 6 injections was carried out for each sample) with different operators in order to evaluate the reproducibility of the analysis. Prior to each series of injections, the membrane was replaced and the detector was recalibrated.

SEC experiments

The SEC system was based on an on-line ERC 3112 degaser (ERC, Saitama, Japan), a Waters 515 pump (Waters Corporation, Milford, USA) and 3 MIXED-A columns in series: 2 styragel columns (porosity 20 μm , 300 mm $\times$ 7.5 mm I.D.) from Waters and 1 PLgel column (porosity 20 μm , 300 mm $\times$ 7.5 mm I.D.) from Varian (Varian, Walnut Creek, USA). All the columns were kept in an oven at 45°C. The detectors were an 18-angle multi-angle light scattering detector (Dawn DSP model, Wyatt Technology, Santa Barbara, USA) and a refractive index detector (Optilab rEX model, Wyatt Technology). Data from angles 5 to 16 were collected and processed with Astra software version 5.3.1 (Wyatt Technology) according to the Berry method with a 2nd order polynomial model. Similarly to the AF4 injection series, each sample triplicate was injected once. Two series were carried out (i.e. a total of 6 injections carried out for each sample).

Theory/calculation

Light scattering theory

For the Berry method with a 2nd order polynomial fit, $[Kc/\Delta R(\theta)]^{1/2}$ is plotted against $\sin^2(\theta/2)$. According to the light scattering theory, this plot makes it possible to determine M_{wi} and R_{gi} for each slice of the fractogram from AF4 or the chromatogram from SEC, respectively, according to equation 1.

$$\left[\frac{Kc}{\Delta R(\theta)} \right]_i^{1/2} = \left[\frac{1}{M_{wi}} + \frac{16\pi^2}{3\lambda_0^2} \frac{\langle R_g^2 \rangle_i}{M_{wi}} \sin^2 \left(\frac{\theta}{2} \right) \right]^{1/2} \quad \text{Equation 1}$$

where K is an optical constant
 c is the solute concentration (in g.mL⁻¹)
 θ is the scattering angle
 $\Delta R(\theta)$ is the excess Rayleigh ratio, the ratio of scattered and incident light intensity
 M_{wi} is the weight-average molar mass of the solute
 λ_0 is the wavelength of the laser beam in a vacuum (in nm)
 R_g is the gyration radius of the solute (nm)

The optical constant K is given by equation 2.

$$K = \frac{4\pi^2 n_0^2}{N_A^2} \left(\frac{dn}{dc} \right)^2 \quad \text{Equation 2}$$

where n_0 is the refractive index of the solvent
 N_A is Avogadro's number
 $\left(\frac{dn}{dc} \right)$ is the differential refractive index increment of the polymer in the solvent used

For detailed information about the light scattering theory, refer to [27,28].

Molar mass and radius definitions

The different molar masses calculated and presented are defined as:

$$M_n = \frac{\sum_i N_i M_i}{\sum_i N_i} \quad M_w = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} \quad M_z = \frac{\sum_i N_i M_i^3}{\sum_i N_i M_i^2} \quad \text{Equation 3, 4 \& 5}$$

Where N_i is the number of polymers with molecular weight M_i
 The radius of gyration ($\langle R_g \rangle$ or R_z) or root mean square radius (r.m.s) is defined for a non-rigid particle as:

$$\langle R_g \rangle = \frac{\sum_i c_i M_i \langle R_g^2 \rangle_i}{\sum_i c_i M_i} \quad \text{Equation 6}$$

Gel content calculation

In order to evaluate the amount of gel retained by the 1 μm filter, the whole peak from the concentration detector (refractive index detector) observed in SEC or AF4 separation was integrated using 0.13 as the value of dn/dc [29]. This gel content was called the “filtrate gel on 1 μm ” or $Gel_{>1\mu}$. In previous papers this quantity of aggregates (macro and micro) was called “total gel”. However, as shown by Kim *et al.* [3] and as we will see later in this paper, a not insubstantial quantity of microaggregates passes through the 1 μm filter. As a consequence, the real total gel (G_T) is:

$$G_T = Gel_{>1\mu} + Gel_{<1\mu} \quad \text{Equation 7}$$

$Gel_{<1\mu}$ is the quantity of microaggregates with a size smaller than 1 μm . $Gel_{<1\mu}$ cannot be determined by SEC-MALS, except by treating the columns with tetrabutylammonium bromide (TBABr) as shown previously [3]. This procedure is rather cumbersome, whereas it will be shown that $Gel_{<1\mu}$ can easily be estimated using AF4 analysis of NR samples. Indeed, by knowing the initial concentration of the sample before filtration, it is possible to determine from the fractogram (DRI signal) the concentration of the two populations in solution after filtration: polyisoprene chains and microaggregates smaller than 1 μm .

Thus:

$$Gel_{>1\mu\text{m}} = \frac{c_1 \times 100}{c_0} \quad \text{Equation 8}$$

232
$$Gel_{<1\mu m} = \frac{C_2 \times 100}{C_0} \quad \text{Equation 9}$$

233

234 Where C_0 is the initial concentration of the analysed sample, C_1 the concentration of the
 235 analysed sample passing through the 1 μm filter (polyisoprene chains + microaggregates) and
 236 C_2 the concentration of only the microaggregates passing through the 1 μm filter. C_1 can be
 237 determined either by SEC or AF4 by integrating the whole peak of the concentration detector
 238 (DRI). C_2 can be calculated only by AF4 integrating the part of the DRI peak containing
 239 microaggregates.

240

241

Results & Discussion

Qualitative description of fractograms

SEC-MALS profiles for the different samples are presented in Figure 1. M_w variations started with a linear decrease corresponding to a normal elution from large chains to small chains but displayed a deviation of the slope of this curve for samples containing gel (i.e. Nippol, M121 and M160, figure 1B, C & D). As described in the introduction and as previously detailed [3], SEC separation of rubber generally presents this abnormal elution due to co-elution of delayed large macromolecules, assumed to be microaggregates ($GEL_{<1\mu}$), with the small chains of poly(*cis*-1,4-isoprene). This particular elution was highlighted when either a change or an inversion of the slope presenting the variation in weight-average molar mass versus elution volume occurred. Regarding the detectors, the differential refractive index (DRI) signal during the SEC elution profile corresponded to a main Gaussian peak (with slight back-tailing) for Nippol and M121, and to a bimodal molar mass distribution for the IR307 and M160 samples. The LS signal was more unimodal with back-tailing varying depending on the sample.

Figure 1

Unlike SEC, AF4 fractionated the macromolecules from small to large ones and the peak shapes appeared quite different. For poly(*cis*-1,4-isoprene) with gel (i.e. Nippol 2200, M160 and M121), the shape of the LS fractograms did not correspond to a Gaussian peak. The fractograms displayed a long front tailing, (i.e. a long and low signal increase) and an abrupt increase in the LS signal from 25 to 30 min (corresponding to a crossflow ranging from 0.5 to

0.3 mL.min⁻¹) (Figure 2). For the IR307 sample, the fractogram exhibited a clearly less pronounced increase in the LS signal (Figure 2A). As the IR307 sample did not contain gel, unlike the Nippol 2200 sample, this abrupt increase in the signal was due to microaggregates, as observed by Andersson *et al.* [30] for AF4 analysis of ethylhydroxyethyl cellulose. For the refractive index detector signal, the behaviour was unfamiliar, with a first peak close to void volume (elution starting at 7 min), corresponding to small chains, followed by either a slight increase in the concentration signal (for IR 307), or a decrease (for Nippol 2200), or quite a constant signal up to the end of the peak (for the two natural samples) (Figure 2). With both the DRI and LS detectors, the fractograms gave a continuous elution whatever the entities eluting, be they random coil or compact microaggregates (sphere-like). However, the evolution of the molar masses (M_{wi}) in line with the elution time was not linear from a qualitative viewpoint (Figure 2). For the IR307 sample, Figure 2A shows a change in slope for an elution time of 17-18 min ($M_{wi} \approx 600 \text{ kg mol}^{-1}$). A clear decrease in the variability of the measured R_{gi} was observed from this elution time ($t_e = 17\text{-}18 \text{ min}$) (Figure 2). Indeed, for the first 18 minutes of the fractogram, R_{gi} values were very dispersed whatever the samples analysed using Berry 2 formalism (Figure 2), compared to SEC-MALS. This high dispersion of R_{gi} may have occurred because the concentrations of the injected solutions were too low ($\approx 1 \text{ mg.mL}^{-1}$) for LS detection or because there was a lack of resolution for small molecule fractionation under our AF4 conditions. This initial slope change in M_{wi} was observed for other samples at times that varied depending on the sample (from 13 up to 17 min). With the Nippol sample, a second marked change appeared in the M_{wi} slope at an elution time of about 31 min (Figure 2B). This abrupt slope change was accompanied by a dramatic increase in the LS signal due to huge entities eluting ($250 < R_{gi} < 1000 \text{ nm}$). This second change in the M_{wi} slope in line with the elution time was not visible for the other synthetic polyisoprene

(IR307), but was visible to a lesser extent for the two NR samples (M121 and M160) at an elution time of about 26-27 min (Figure 2C & D).

Figure 2

The M_{wi} AF4 profile for IR 307 confirmed the presence of only one population of chains with a M_{wi} ranging from 60,000 g.mol⁻¹ to 5 million g.mol⁻¹ (Figure 2A). Conversely, the Nippol AF4 profile clearly showed the presence of 2 distinct populations (Figure 2B). The first population was composed of isolated polyisoprene chains and the second one (starting at 32 min) was composed of microaggregates with sizes under 1 μ m (Gel_{<1 μ}) with a drastic increase in the M_{wi} slope. For the two NR samples (M121 and M160) (Figure 2C and 2D), the M_{wi} profiles were quite different from that for Nippol. Isolated polyisoprene chains seemed to elute first up to about 27 min. After this elution time, the M_{wi} profile exhibited an increase in slope (from 27 to about 35 min), reaching a quasi-plateau (from 35 min). This behaviour suggests that this population was quite monodisperse. These two parts could be attributed to co-elution of isolated polyisoprene chains and Gel_{<1 μ} in the first case and Gel_{<1 μ} only for the plateau.

Large differences were observed for the $M_{wi}=f(V_e)$ and $R_{gi}=f(V_e)$ slopes, the sizes (R_{gi}) and the molar masses (M_{wi}) of the second populations for Nippol compared to the two natural polyisoprene samples (Figure 2). These results tend to confirm that the gel was not intrinsically the same for natural and synthetic polyisoprene. Indeed for NR, the radius for the Gel_{<1 μ} was about 150 nm for a molar mass close to 15 million g.mol⁻¹ whereas for Nippol for the same molar mass the radius was about 240 nm (i.e. 60% higher), as illustrated in Figure 3. Moreover, there was no “plateau effect” for Nippol compared to the NR samples. Thus, with a lower R_{gi} for the same M_{wi} , the microaggregates in the NR samples seemed more compact

than in the Nippol synthetic polyisoprene. However, some of the difference observed in the slopes may have been due to an underestimation of M_{wi} for the microaggregates of the NR samples. Indeed, it cannot be ruled out that the dn/dc of NR microaggregates is not constant. Changes in the non-isoprene composition (lipids and/or proteins) of microaggregates cannot be excluded. A change in the proportion of non-isoprene compounds versus polyisoprene in the microaggregates could lead to a change in the dn/dc and so in the M_{wi} determined.

Figure 3

One of the main difficulties encountered with AF4 fractionation is to define a correct and unbiased integration range. For our data, the integration range for average molar mass and radius of gyration assessment was started when the M_{wi} and R_{gi} signals were stabilized (out of void peak influence) and stopped just before the end of the peak (shown as the red box in figure 4 for M160, for example). This choice of integration area was made in order to have results including almost all the peak surface and to avoid a decrease in repeatability for the AF4 results due to pre- and post-peak radius and mass dispersion and heterogeneity because of a low LS signal. This method was applied for the AF4 signals from IR307, M160 and M121. On the other hand, the treatment for Nippol 2200 was different. Indeed, the variation in M_{wi} in the second part of Nippol 2200 fractionation was quite high due to the presence of microaggregates with ultra high molar masses (over 10^{10} g mol⁻¹). Moreover, this final M_w increase was quite noisy and unrepeatable, probably due to the low associated RID signal. In fact, as presented in table 2 for example, increasing the Nippol 2200 integration range (from 9.5 - 35 min to 9.5 - 40 min), drastically increased the average molar masses, but also the standard deviation. Lastly, a 7% increase in RID integration area (hence in quantity) led to an increase of 4 orders of magnitude for the M_w and resulted in an increase in the standard

deviation from 25% to 71%. Consequently, for Nippol, the integration range was shortened, compared to the other samples, to avoid excessive variability in the results.

Table 2

In order to quantify aggregates exceeding 1 μm ($\text{Gel}_{>1\mu}$) (estimation of C_1 in eq. 9), the whole peak was integrated (green box in figure 4). The quantity of microaggregates smaller than 1 μm ($\text{Gel}_{<1\mu}$) (estimation of C_2 in eq. 10) was calculated according to a third integration range (the blue box in figure 4) starting from the middle of the zone we considered as a mixture of polyisoprene chains and microaggregates (data $R_g=f(V_e)$) up to the end of the peak.

Figure 4

Comparison of data between SEC-MALS and AF4-MALS

Average molar masses

M_n , M_w and M_z were calculated from the SEC and AF4 results for each sample. The results are presented in Figure 5.

Figure 5

The number average molar mass M_n did not present significant differences whether determined by SEC or AF4 (Figure 5). The ratio of M_n obtained by AF4 to that obtained by SEC was ranged between 0.85 and 1.0. Unlike M_n , M_w and M_z were different, with a higher M_w and M_z obtained for AF4. As described previously, these larger M_w and M_z can be

explained by the presence of large microaggregates not observed with SEC. This observation is confirmed by the fact that for IR 307 (poly(*cis*-1,4-isoprene) without gel), M_w and M_z exhibited no significant difference between SEC and AF4 results. The difference between AF4 and SEC in terms of average molar masses was, as expected, higher for M_z than for M_w . The M_w ratios ($M_{w-AF4}:M_{w-SEC}$) ranged from 1.0 to 4.3 (for IR 307 and M121 respectively) whereas the M_z ratios ($M_{z-AF4}:M_{z-SEC}$) ranged from 1.0 to 18.6 (for IR 307 and Nippol respectively). For the three average molar masses considered (i.e. M_n , M_w or M_z), the AF4 results showed greater heterogeneity than for the SEC results, with high standard deviations. This lower reproducibility and repeatability was mainly due to the difficulty in defining the integration range, as previously explained, but also to the substantial variability generated by the refractive index detector baseline position. Indeed, the AF4 principle resulted in some noise, deviations and jumps in the DRI baseline and thus to some doubts / uncertainties on the baseline position, despite the blank subtraction. The large M_z difference (from AF4 to SEC) and RSD for Nippol was due to the large amount of microaggregates with ultra high molar masses and to the poor repeatability in the high mass range, as explained later on.

Determination of gel rates

The gel rates in the samples were calculated after SEC and AF4 analysis. $Gel_{>1\mu}$ was calculated for both the SEC and AF4 analyses whereas $Gel_{<1\mu}$ was only calculated after AF4 analysis. Figure 6 presents the $Gel_{>1\mu}$ rate after SEC and AF4 analyses. For the IR 307 and Nippol samples, the $Gel_{>1\mu}$ rate did not display any significant difference (no gel for IR307 and average $Gel_{>1\mu}$ slightly higher for Nippol with AF4). For the two NR samples, only M160 exhibited a significant difference in the $Gel_{>1\mu}$ rate between SEC and AF4 determination (23.8% for SEC and 17.2% for AF4). For M121, the lack of significant difference was due to the high variance of the AF4 result. The $Gel_{<1\mu}$ calculation was estimated at 9.5% for Nippol,

27% for M160 and 29% for M121 (no Gel_{<1μ} for IR 307). These large quantities of Gel_{<1μ} explained the large differences observed for M_w and M_z in the NR samples and the Nippol sample (higher M_w and M_z for AF4 analyses compared to SEC). However, as a result of such a large quantity of Gel_{<1μ} for the NR samples, assumed to be “lost” in not insubstantial proportions in SEC, the Gel_{>1μ} rate should have been much lower with AF4 compared to SEC for Nippol, M160 and M121. The slight difference observed between AF4 and SEC for the Gel_{>1μ} rate could be explained either by the large measurement variability for AF4, or by an overestimated Gel_{>1μ} calculation in AF4 analysis (potentially due to concentration peak area determination and therefore to blank subtractions). For the Gel_{<1μ} calculation, Kim *et al.* [3] obtained Gel_{<1μ} values close to 10% (calculated by SEC after ionic surfactant treatment of the columns) for NR samples similar to M160 and M121, meaning a difference with our Gel_{<1μ} values of about 15%.

Figure 6

Comparison of radii of gyration

As for the average molar masses, the R_g values obtained with AF4 were higher than those obtained with SEC, except for the IR307 sample, whose R_g was the same for both separation techniques (see table 3). Nevertheless, the differences in R_g obtained with the two techniques were less pronounced than for M_z. This was due to the narrower radius of gyration size range observed (typically varying from 20 to 200 nm) compared to the 10² - 10³ order of magnitudes for the masses over the whole fractionation (up to 10⁵ for Nippol). The variation in R_{gi} depending on the elution time with AF4 displayed a similar behaviour to that for M_{wi} (Figure 2). As the variation in R_{gi} was less significant than for M_{wi}, it implies that the material was becoming increasingly compact (greater increase for masses than for radii) towards the

416 end of the elution, especially for the second population, as previously illustrated in figure 3.
417 This observation confirms the presence of compact microaggregates highlighted by Kim *et al.*
418 [\[3\]](#).

419

420 *Table 3*

421

Conclusions

This work demonstrates the ability of AF4 to fractionate natural and synthetic poly(*cis*-1,4-isoprene). Distinct populations (characterized by a clear slope change in M_{wi} variation) corresponding to isolated polyisoprene chains and microaggregates smaller than $1\ \mu\text{m}$ ($\text{Gel}_{<1\mu}$) were detected. Average molar masses were determined and compared with those obtained by SEC. Similar M_n values were obtained but large differences were observed for M_w and M_z . These differences could be explained by a microgel population observed in AF4 but not during SEC separation. Moreover, microaggregates in the NR samples exhibited quite a different structure, appearing more compact than the microaggregates in the Nippol synthetic polyisoprene. Some problems (difficulties in defining the baseline, DRI signal jumps, etc.) due to the use of DRI with AF4 were encountered, in particular for gel rate determination. It seems obvious that some analytical developments are needed to optimize fractionation, increase the resolution for small polyisoprene chains and enable better reproducibility. However, these results are promising and microgel ($\text{Gel}_{<1\mu}$) can be considered for further individual studies and physico-chemical characterization.

438 **Acknowledgements**

439 The authors gratefully thank Emilie Croizat and Christine Char for their valuable contribution
440 to this project. A4F-MALS and SEC-MALS analyses were carried out on the LipPol-Green
441 platform (<http://www.supagro.fr/plantlippol-green/>). We kindly thank CIRAD DRS and
442 Agropolis fondation ([http:// http://www.agropolis-fondation.fr](http://www.agropolis-fondation.fr)) for financial support, and the
443 Hubert Curien Programme for funding Miss Chalao Thepchalerm's scholarship.

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492

Figure 1

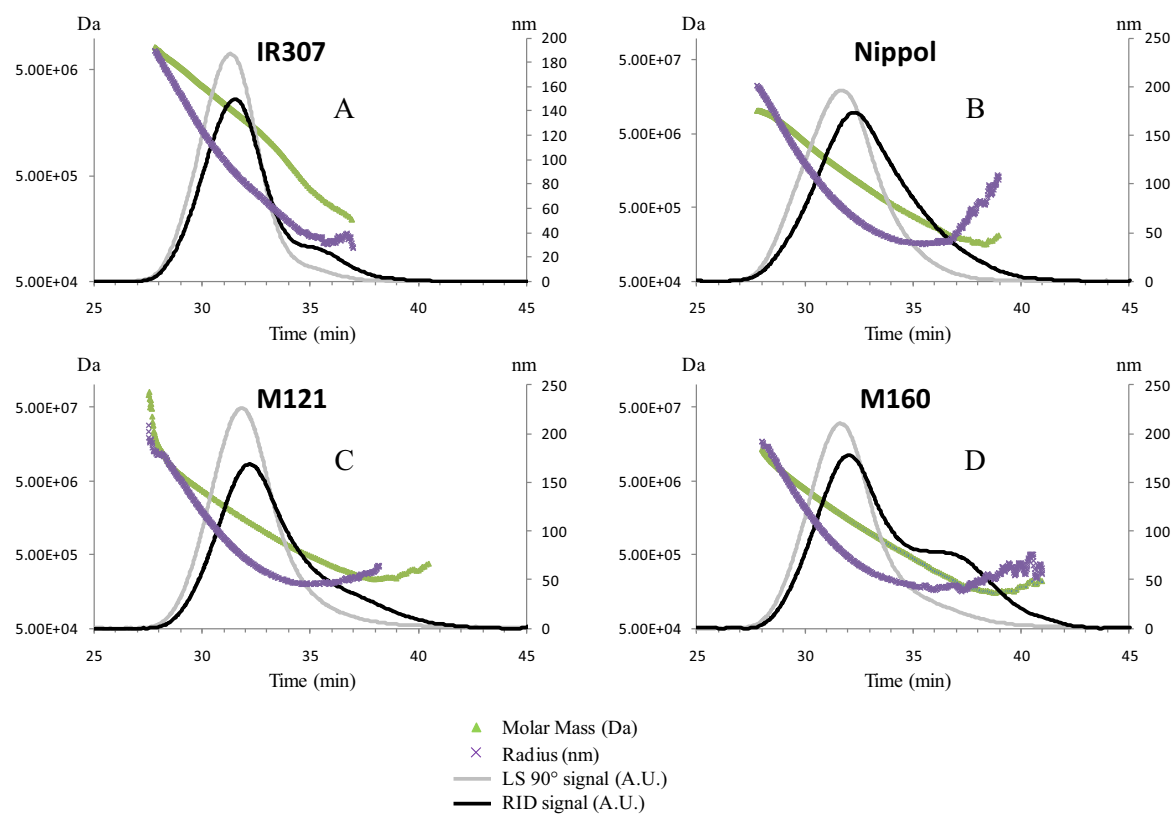


Figure 1: Chromatograms (from the RID and LS detector at 90°) and variation in R_g and M_i depending on the elution time during SEC analysis

Figure 2

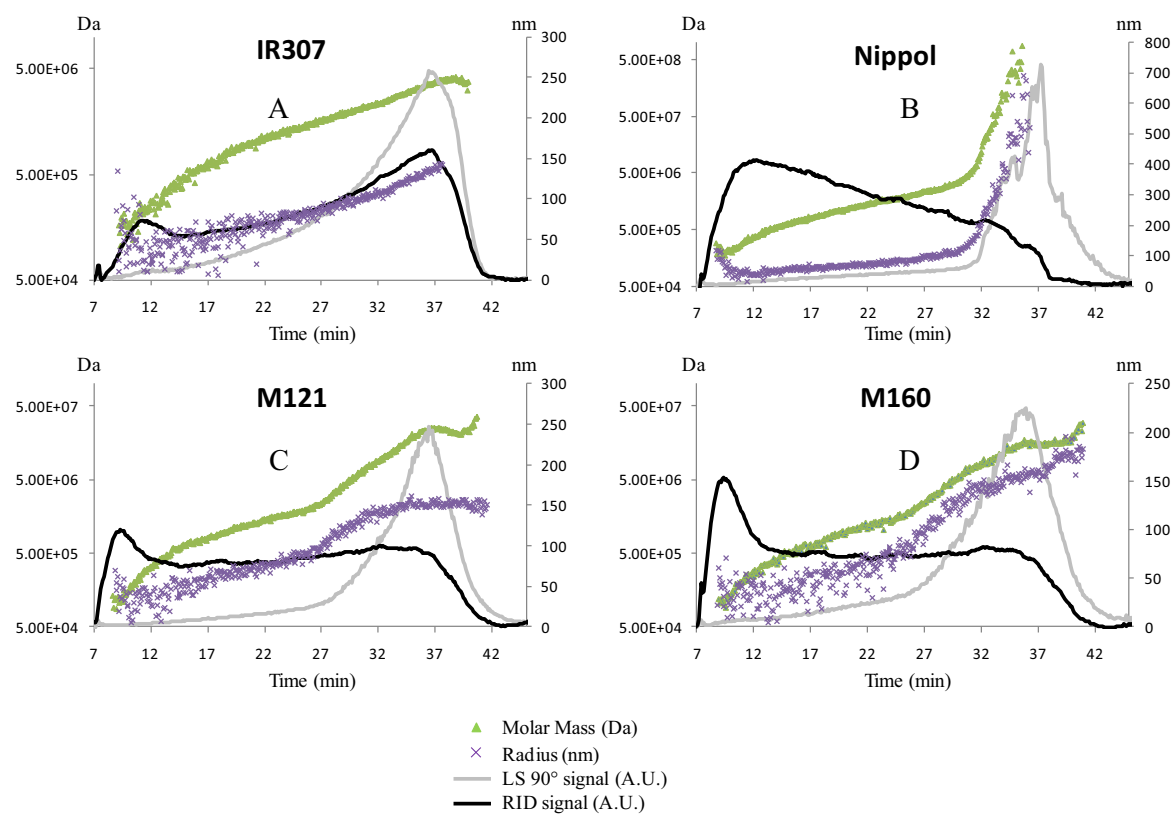


Figure 2: Fractograms (from the RID and LS detector at 90°) and variation in R_g and M_i depending on the elution time during AF4 analysis

Figure 3

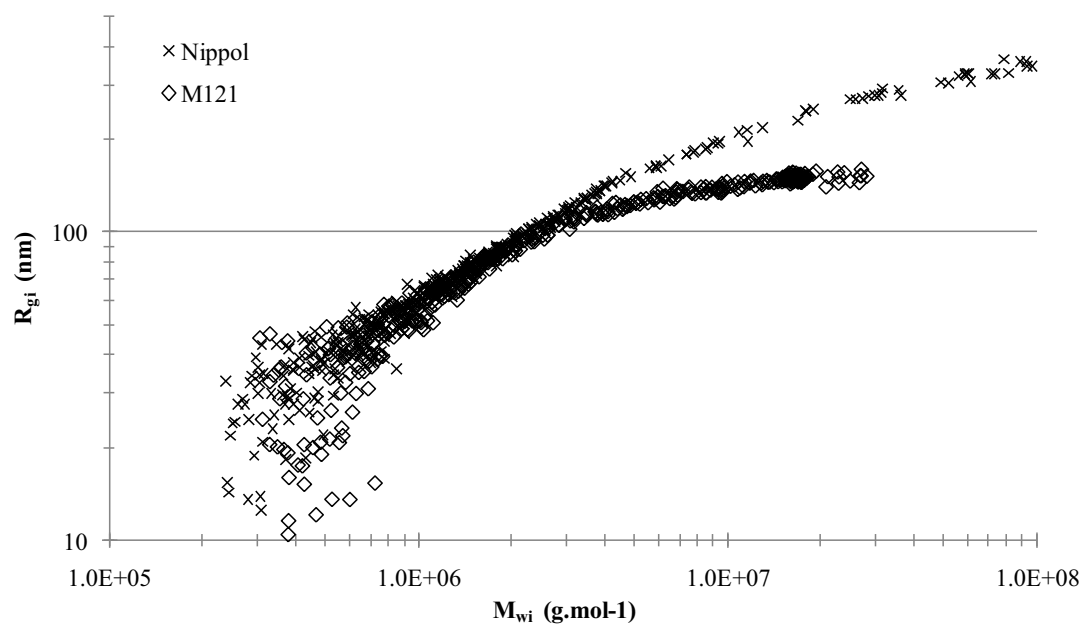


Figure 3: Variation of the radius of gyration (R_{gi}) depending on molar masses (M_{wi}) for the Nippol 2200 and M121 samples.

Figure 4

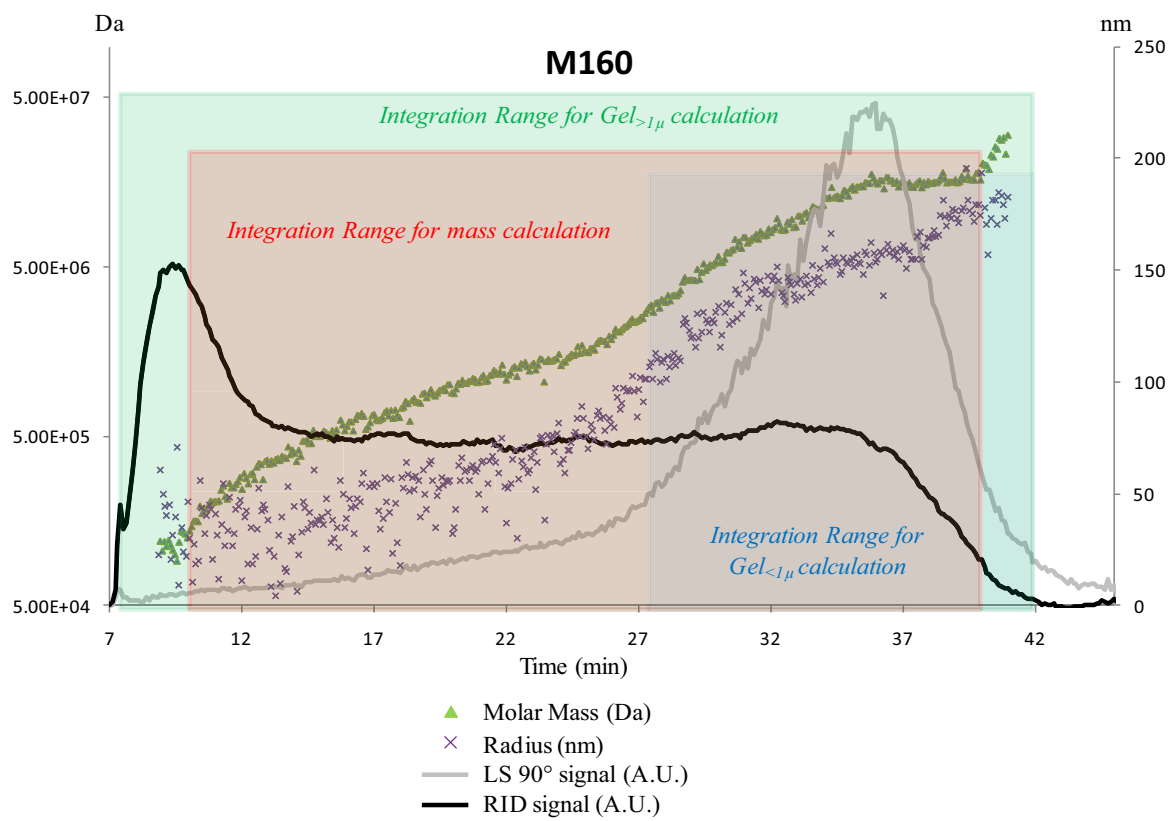


Figure 4: Determination of the AF4 integration range for the M160 sample

Figure 5

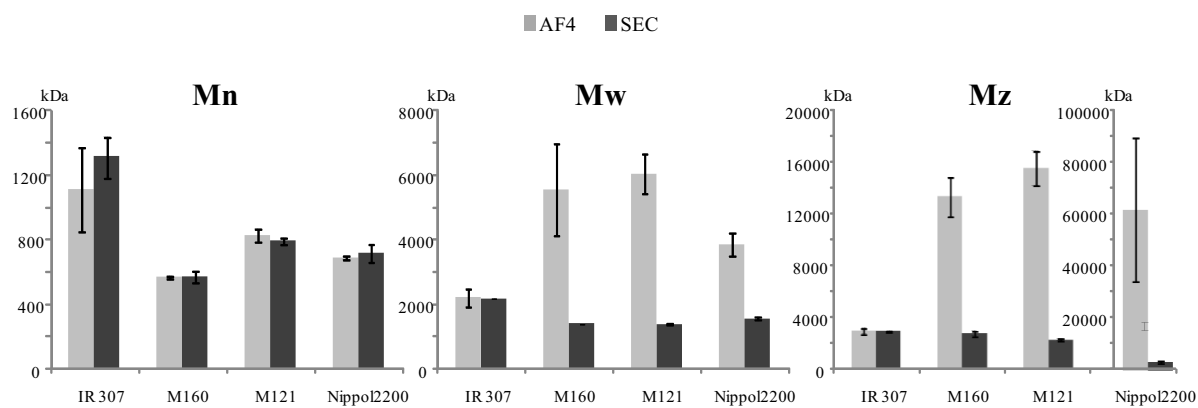


Figure 5: Comparison of masses obtained by SEC and AF4

Figure 6

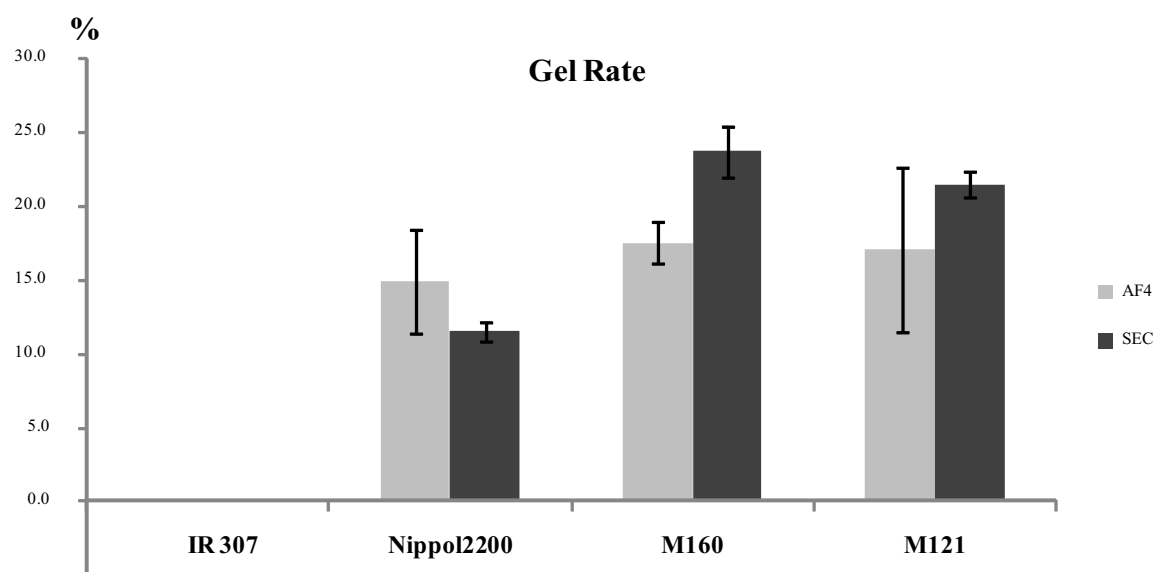


Figure 6: Comparison of filtrate gel on 1 μm ($\text{Gel}_{>1\mu}$) rate calculated by AF4 and SEC

<i>Step</i>	<i>Duration (min)</i>	<i>Crossflow (mL.min⁻¹)</i>	<i>Type of rate</i>
<i>1</i>	<i>1.5</i>	<i>1</i>	<i>Constant</i>
<i>2</i>	<i>30</i>	<i>From 1 to 0.08</i>	<i>Linear</i>
<i>3</i>	<i>9</i>	<i>0.08</i>	<i>Constant</i>
<i>4</i>	<i>7</i>	<i>0</i>	<i>Constant</i>

Table 1: AF4 crossflow programme during elution

	integration start	integration end	M _n (Da)	RSD	M _w (Da)	RSD	M _z (Da)	RSD
Nippol 2200	9.5 min	35 min	6.95E+05	4%	3.61E+06	25%	4.24E+07	62%
	9.5 min	40 min	7.48E+05	4%	9.36E+10	71%	1.40E+14	81%

 Integration : +7%
of surface area

Table 2: Nippol 2200 mass calculation (M_n, M_w and M_z with respective RSD from one AF4 series) with different integration ranges

		R_g (nm)	SD (nm) (1)	CV (1)
IR307	AF4	115.8	3.8	4.0%
	SEC	112.1	1.4	1.3%
Nippol	AF4	249.9	28.8	28.8%
	SEC	108.5	4.2	3.9%
M160	AF4	136.5	7.0	4.3%
	SEC	102.8	2.3	2.2%
M121	AF4	133.5	7.8	3.4%
	SEC	92.5	2.4	2.6%

(1) SD: standard deviation, CV: coefficient of variation ((SD/mean) x 100)

Table 3: R_g determined by AF4 and SEC

Manuscript Number:

Title: Surface-quaternized chitosan particles as an alternative and effective organic antibacterial filler

Article Type: Full Length Article

Keywords: chitosan; particle; heterogeneous quaternization; minimum inhibitory concentration.

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Abstract: Taking advantage of the large surface area that is covered with permanent positive charges of quaternary ammonium entities, this research aimed to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical devices, health and textile industries. The particles were formulated by ionic crosslinking of chitosan with tripolyphosphate followed by quaternization that was conducted under heterogeneous condition via either direct methylation or reductive N-alkylation with a selected aldehyde followed by methylation. Sub-micron, spherical, and positively charged quaternized chitosan particles were formed, as determined by ¹H-NMR, FT-IR, PCS, and TEM analysis. Antibacterial activity tests performed by viable cell counts suggested that all quaternized chitosan particles exhibited superior antibacterial activity against the model Gram-positive bacteria, *S. aureus*, as compared to the native chitosan particles at neutral pH. Only some quaternized chitosan particles, especially those having a high charge density and bearing large alkyl substituent groups, were capable of suppressing the growth of the model Gram-negative bacteria, *E. coli*. The inhibitory efficiency of the quaternized chitosan particles was quantified in terms of the minimum inhibitory concentration (MIC).



June 12, 2011

Dear Sir/Madam:

I would like to send a manuscript, entitled “**Surface-quaternized chitosan particles as an alternative and effective organic antibacterial filler**” to be considered for publication as a full paper in **Colloids and Surfaces B: Biointerfaces**. On behalf of all authors, namely, Oraphan Wiarachai, Nuttha Thongchul, Suda Kiatkamjornwong, Voravee P. Hoven, I hereby confirm that this manuscript or its contents in some other form has not been published previously by any of the authors and/or is not under consideration for publication in another journal at the time of the submission.

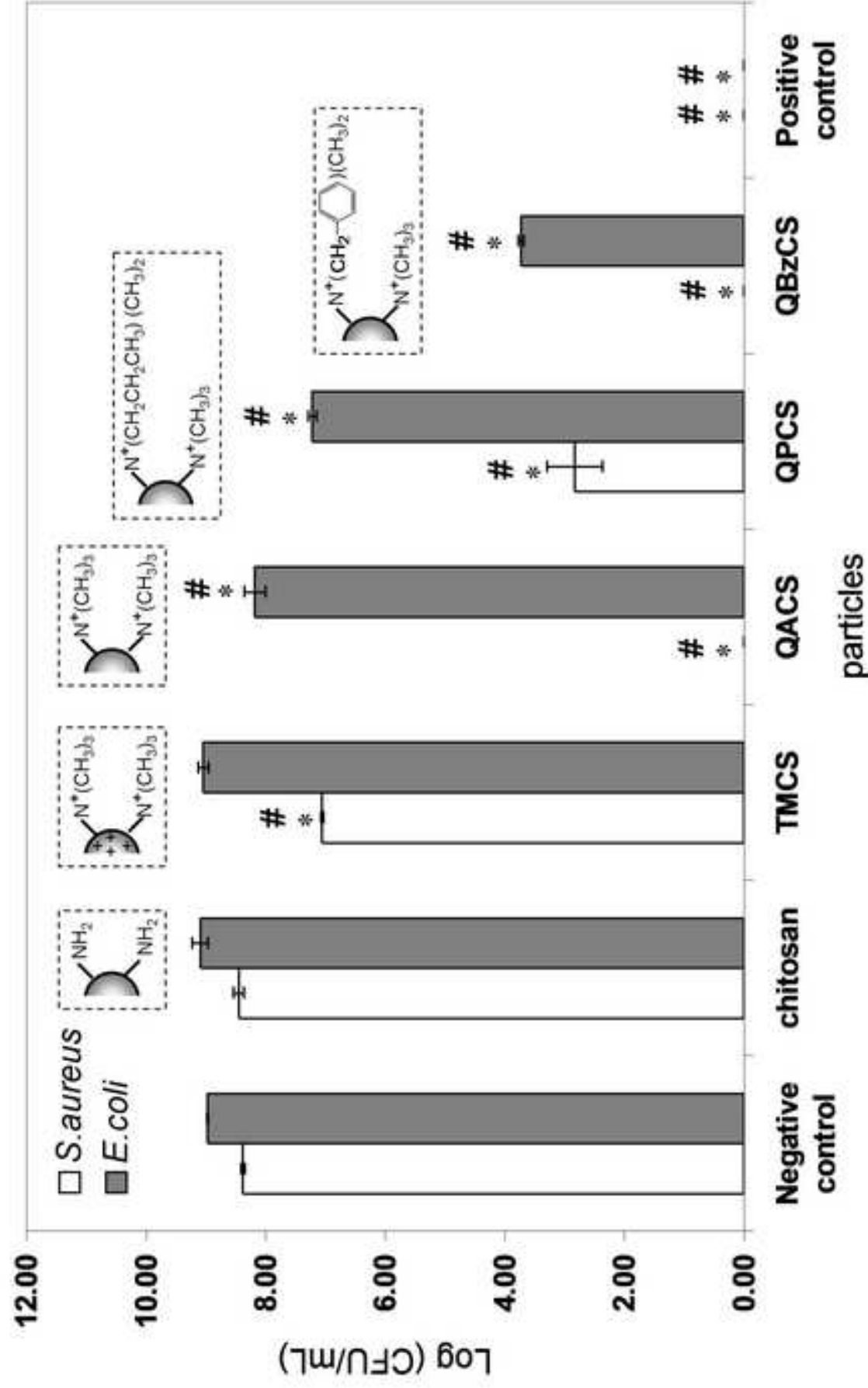
This work introduces surface-quaternized chitosan particles as an effective, environmentally friendly, organic antibacterial filler that may be incorporated into materials that require antibacterial activity. Based on the minimum inhibitory concentration, it was found that some surface-quaternized chitosan particles are more effective in inhibiting the bacterial growth than the quaternized chitosan solution. The fact that the heterogeneous quaternization used for the preparation of surface-quaternized chitosan particles can simply be done without the requirement for tedious purification process opens up an opportunity to produce such effective antibacterial filler at a large scale and that is very beneficial from industrial perspective.

The authors believe that the content of this contribution would be of interest to **Colloids and Surfaces B: Biointerfaces** audiences. Should there be any further information which may be required or if any problems arise, please contact me at the address shown below.

Sincerely yours,

Vp. Hoven

Voravee P. Hoven, Ph.D.
Corresponding Author



[* $p < 0.01$ (compared with the negative control), # $p < 0.01$ (compared with the chitosan particles)]

Research highlights

- Quaternized chitosan particles prepared by heterogeneous quaternization exhibited significantly higher antibacterial activity against *S. aureus* (Gram positive bacteria) and *E. coli* (Gram negative bacteria) than the particles prepared by homogeneous quaternization.
- Only some quaternized chitosan particles, especially those having a high charge density and bearing a large alkyl substituent, were capable of suppressing the growth of *E. coli*, the Gram-negative bacteria.

Surface-quaternized chitosan particles as an alternative and effective organic antibacterial filler

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ABSTRACT

Taking advantage of the large surface area that is covered with permanent positive charges of quaternary ammonium entities, this research aimed to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical devices, health and textile industries. The particles were formulated by ionic crosslinking of chitosan with tripolyphosphate followed by quaternization that was conducted under heterogeneous condition via either direct methylation or reductive *N*-alkylation with a selected aldehyde followed by methylation. Sub-micron, spherical, and positively charged quaternized chitosan particles were formed, as determined by ¹H-NMR, FT-IR, PCS, and TEM analysis. Antibacterial activity tests performed by viable cell counts suggested that all quaternized chitosan particles exhibited superior antibacterial activity against the model Gram-positive bacteria, *S. aureus*, as compared to the native chitosan particles at neutral pH. Only some quaternized chitosan particles, especially those having a high charge density and bearing large alkyl substituent groups, were capable of suppressing the growth of the model Gram-negative bacteria, *E. coli*. The inhibitory efficiency of the quaternized chitosan particles was quantified in terms of the minimum inhibitory concentration (MIC).

Keywords: chitosan; particle; heterogeneous quaternization; minimum inhibitory concentration

1. Introduction

Incorporation of antibacterial fillers is an efficient approach to introduce antibacterial activity to materials. There are many types of filler that can be incorporated into the materials to induce their antibacterial activity such as antibiotic [1], nitric oxide [2], halogen species [3, 4], biocide [5] and heavy metal [6-8]. From several types of antibacterial fillers, heavy metal in the form of silver nanoparticles (AgNPs), are well recognized to exhibit an effective antibacterial activity and are widely used in many applications such as medical devices [9], paint [10], coating [11] and textiles [12]. Recently, it has been reported that the AgNPs could affect human tissues from diverse routes of exposure including the respiratory system, skin, and gastrointestinal tract [13]. It has been proposed that AgNPs may interact with thiol groups of proteins and enzymes within mammalian cells, some of which are key components in the antioxidant defense mechanism of the cell. Inhibition of this mechanism would then result in an increase in the concentration of reactive oxygen species that can alter or damage the function of the cells. Also, it has been found that the AgNPs can be released from commercial clothing (socks) into the wash water [14], and so could increase the risk of environmental contamination. Therefore, it is highly desirable if the antibacterial fillers can be developed from natural, nontoxic and environmentally friendly material.

Chitosan is a natural biopolymer derived from the deacetylation of chitin, an abundantly available biopolymer found in the exoskeletons of insects, the shell of crustaceans, and fungal cell walls. It is nontoxic, biodegradable, biocompatible and possesses antibacterial properties [15]. In fact, the potential of chitosan particles as an effective antibacterial agent/filler has been realized and continuously reported. Early work by Qi and co-workers demonstrated that chitosan in the form of nanoparticles exerted a higher antibacterial activity against *E. coli*, *S. choleraes*, *S. typhimurium*, and *S. aureus* than chitosan solution, and it was proposed that the greater surface area of the particles provided a

more intimate contact with the surface of bacterial cells [16]. Ye and co-workers, on the other hand, have shown that cotton fabrics coated with core-shell particles, having poly(*n*-butyl acrylate) as the core and chitosan as the shell, exhibited an excellent antibacterial activity against *S. aureus* with a reduction in viable bacteria numbers of more than 99% [17].

In general, chitosan would exhibit its antibacterial activity only in acidic medium, due to the poor solubility of chitosan above its pKa (pH 6.5). Quaternization of the amino groups to introduce permanent positively charged quaternary ammonium groups has been recognized as a potential way to enhance the antibacterial activity of chitosan over a broader pH range. The reaction can simultaneously introduce the permanent positive charge and hydrophobicity, the two parameters that are believed to affect the antibacterial activity [18-20]. Synthetic routes and applications of quaternized derivatives of chitosan have been recently reviewed and published [21, 22], whilst the application of quaternized chitosan particles has been reported. For example, particles fabricated from chitosan that was formerly quaternized by hexyl bromide were incorporated as bactericidal fillers in poly(methyl methacrylate) bone cement and tested against *S. aureus* and *S. epidermidis* under a neutral pH environment [23].

In order to promote ionic interactions with the negatively charged components of the bacterial cell membrane, the incorporation of metal ions [24-26] or cationic polymer [27] into chitosan particles has also been recently reported as an alternative approach. Qi and co-workers have suggested that the antibacterial activity can be enhanced by incorporating Cu²⁺ into chitosan particles [25]. Later, Du and co-workers have shown that chitosan particles loaded with various metal ion such as Ag⁺, Cu²⁺, Zn²⁺, Mn²⁺, Fe²⁺, exhibited higher antibacterial effect against *E. coli*, *S. choleraesuis*, and *S. aureus* than chitosan particles [24, 26]. In addition, Inphonlek and coworkers have found that core-shell nanoparticles having poly(methyl methacrylate) core coated with polyethyleneimine, and chitosan-mixed-

polyethyleneimine shells exhibited higher antibacterial activity against *S. aureus* and *E. coli* than chitosan nanoparticles [27].

Here in this research, we attempt to develop an environmentally friendly, organic antibacterial filler from chitosan particles. The permanent positive charges in the form of quaternary ammonium groups were introduced to the surface of pre-fabricated chitosan particles under heterogeneous condition via direct methylation or reductive *N*-alkylation using two different aldehydes, propionaldehyde and benzaldehyde, followed by methylation with methyl iodide. This method has the advantage that it can be performed conveniently in the absence of tedious purification process that is generally required for homogeneous quaternization. The success of this type of method for enhancing antibacterial activity especially in a neutral pH environment has been previously reported for the chitosan films [28]. And the fact that the addition of cationic metal species or polymers is no longer necessary truly simplifies the fabrication method.

2. Materials and methods

2.1. Materials

Chitosan flake (DAC 85%, $M_V = 45,000$ Da) was purchased from Seafresh Chitosan (Lab) Co., Ltd (Thailand). Propionaldehyde, methyl iodide (CH_3I), sodium borohydride (NaBH_4), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium iodide (NaI) and tripolyphosphate (TPP) were purchased from Fluka (Switzerland). Acetic acid, benzaldehyde, ethanol (EtOH), and methanol (MeOH) were purchased from Merck (Germany). Dialysis membrane (cut-off molecular weight of 12,400 g/mol) was purchased from Sigma (USA). All reagents and materials were of analytical grade and used without further purification. *E. coli* and *S. aureus* were provided by National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were

purchased from Difco (USA). Mueller Hinton agar plates were supplied by Department of Medical Science, Ministry of Public Health, Thailand. Ultrapure distilled water was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step.

2.1. Preparation of surface-quaternized chitosan particles

Chitosan particles were prepared using TPP as a crosslinking agent according to the published procedure by Qi *et al.* [16] Preparation of quaternized *N*-alkyl chitosan particles were done following the method of Vallapa *et al.* [28]. In brief, an anhydrous methanol solution of a selected aldehyde (10 mL) having a desired concentration was added into a flask containing chitosan particles (0.03 g). After stirring for a given time at ambient temperature (~ 28 - 30°C), NaBH_4 (0.3 g, 0.8 mol) was added into the reaction mixture and the solution was stirred for 24 h. The resulting *N*-alkyl chitosan particles were then isolated by centrifugation at 12,000 rpm for 30 min. The supernatant was discarded and the particles were centrifugally washed as above with methanol three times and then dried *in vacuo*. An anhydrous methanol solution (10 mL) containing NaI (0.30 g, 2 mmol) and NaOH (0.13 g, 3 mmol) was then added into a flask containing chitosan or *N*-alkyl chitosan particles (0.03 g) and CH_3I was then added via syringe. The reaction mixture was stirred at 50°C for a given time. The particles were isolated and purified as above mentioned.

2.2. Characterization of surface-quaternized chitosan particles

Fourier Transform-Infrared (FT-IR) spectra were collected using a Nicolet Impact 410 FT-IR spectrometer. All samples were prepared as KBr pellets. All ^1H NMR spectra were recorded in a 1% (v/v) solution of $\text{CF}_3\text{COOH}/\text{D}_2\text{O}$ or D_2O using a Varian model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts

(δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference. The size and the morphology of all particles were examined by transmission electron microscope (TEM, Model JEM-2100, Japan) with the average diameter calculated using Semafore software and reported from the measurement of 100 randomly selected particles. The ζ -potential values of the particles were determined using a Nanosizer Nano-ZS (Malvern Instruments, UK) at 25°C with a scattering angle of 173°. All data are displayed as the mean $\pm$ one standard deviation and are derived from at least three independent experiments. The data were calculated using the Helmholtz-Smoluchowski equation.

2.8. Evaluation of antibacterial activity

All glasswares used for the tests were sterilized in an autoclave at 121°C for 15 min prior to use. All particles were sterilized by exposing to UV radiation for 60 min prior to the tests. The antibacterial test was performed according to a modified method of Qi *et al.* [16]. Chitosan and quaternized chitosan particles (2.5 mg) were added to 5 mL of MHB in test tubes and aseptically inoculated with 50 μ L of the freshly prepared bacterial suspension in distilled water ($OD_{600} = 0.5$). The pH of the broth was about 7.4. The negative and positive controls were a broth solution alone and a broth solution with ampicillin (50 mg/mL), respectively. After mixing, broth cultures were incubated at 37°C in a shaking incubator (Model G-25, New Brunswick Scientific Co., Inc., USA), at 110 rpm for 24 h. Then, 100 μ L of the bacterial solution was removed and 10-fold serially diluted down to 10⁶-fold the original level. A 100 μ L aliquots of each diluted bacterial suspension was then spreaded onto the MHA plates for total plate counting. After incubating at 37°C for 18 h, the number of colonies, and thus replication competent bacteria, were then counted as a measure of the

assumed viable number of bacteria. The results after correction for the dilution factor were expressed as mean colony forming units per volume (CFU/mL).

To determine the minimum inhibitory concentration (MIC) of all particles, the particles were first suspended in 5 mL of MHB and two-fold serial dilutions prepared. Each set of serial dilutions was then inoculated under aseptic conditions with 50 μ L of the freshly prepared bacterial suspension in distilled water ($OD_{600} = 0.5$). After incubating at 37°C for 24 h, the total plate count enumeration of the CFU/mL was evaluated as described above. The lowest concentration of the particles that inhibited the growth of bacteria was considered as the minimum inhibitory concentration (MIC).

All tests of antibacterial activity were performed in triplicate per sample, upon at least three independent samples and expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 17.0 software. Statistical comparisons made by One-Way Analysis of Variance (ANOVA) with the Least Square Difference (LSD) tests were used for post hoc evaluations of differences between groups. The threshold level for accepting statistical significance was set at $p < 0.01$.

3. Results and Discussion

3.1. Preparation and characterization of surface-quaternized chitosan particles

Chitosan particles were first fabricated by ionic crosslinking between positively charged chitosan and negatively charged TPP. The process was conducted at ambient temperature in the absence of any surfactant or oil phase. The resulting chitosan particles appeared as fine white powder after drying and were insoluble in water, dilute acidic and alkali solutions. As outlined in **Scheme 1**, chitosan particles were then surface-modified by heterogeneous reductive *N*-alkylation with selected aldehyde, namely propionaldehyde and

benzaldehyde which yielded *N*-propyl chitosan particles and *N*-benzyl chitosan particles, respectively. Subsequent methylation with CH₃I thus formed quaternized *N*-propyl chitosan (QPCS) particles and quaternized *N*-benzyl chitosan (QBzCS) particles, respectively. Furthermore, the quaternization (Scheme 1) was also conducted directly on chitosan particles by methylation with CH₃I and gave quaternary ammonium-containing chitosan (QACS) particles as products. The optimized conditions for the preparation of each surface-quaternized chitosan particles via heterogeneous quaternization, so as to yield the highest degree of quaternization in terms of reaction time and reagent concentration were empirically identified (Table S1, Supplementary Material) based on the results from water contact angle and ζ -potential measurements following the criteria as previously described [28]. Another type of quaternized particles designated as *N,N,N*-trimethylchitosan (TMCS) particles were prepared for comparison. Although the quaternary ammonium entity (trimethyl, -N(CH₃)₃) of the TMCS particles is similar to that of the QACS particles, the TMCS particles were prepared differently by ionic gelation of *N,N,N*-trimethylchitosan synthesized homogeneously from chitosan according to the method reported by Sieval *et al.* [29]. In other words, TMCS particles should not only bear positive quaternary ammonium groups at the particles' surface which is the case for the QACS particles, but also inside the particles.

Chemical functionalities of all quaternized chitosan particles were first identified by FT-IR analysis. As shown in Fig. 1, absorption peaks at 1650 and 1544 cm⁻¹ can be assigned to the C=O stretching (Amide I) and N-H bending (Amide II) of the glucosamine unit of chitosan, respectively. The decrement of the N-H bending signal at 1544 cm⁻¹ and the appearance of the C-H deformation signal in a range of 1470-1460 cm⁻¹ in the spectra of all quaternized chitosan particles implied that the amino groups were functionalized by alkyl substituents introduced by either reductive *N*-alkylation/methylation or direct methylation. Nonetheless, the C-H deformation peak belonging to the TMCS particles was obviously weak

as opposed to those observed for other particles implying that there was smaller content of quaternary ammonium groups in the TMCS particles.

The degree of success in the quaternization of chitosan particles was also confirmed by ^1H NMR analysis. As illustrated in Fig.2, the multiplet in the range of $\delta 3.3 - 4.4$ ppm comes from the six carbon atoms at the 2', 3, 4, 5, 6, and 6' positions, while the two singlets at $\delta 1.9$ and $\delta 2.9$ ppm are due to the methyl protons from the acetyl groups (position A) and at the 2 position of *N*-acetyl glucosamine unit, respectively. For the QPCS and QBzCS particles, it should be emphasized that not all of the amino groups of chitosan were alkylated in the first step of reductive *N*-alkylation. As a consequence, the signals of the methyl protons appearing in Fig.2(d-e) would come from methyl groups attached to both alkylated and non-alkylated amino functionalities. The signal at $\delta 3.1-3.3$, $\delta 3.0$, and $\delta 2.7$ ppm can be assigned to the methyl protons of the trisubstituted ($-\text{N}^+(\text{CH}_3)_3$ or $\text{NR}^+(\text{CH}_3)_2$, position B), disubstituted ($-\text{N}(\text{CH}_3)_2$ or $-\text{NR}(\text{CH}_3)$, position C) and monosubstituted ($-\text{NH}(\text{CH}_3)$, position D) amino groups, respectively. The trace signals of methylene protons ($\delta 1.0$ ppm) and methyl protons ($\delta 0.85$ ppm) observed on the spectrum of QPCS particles (Fig.2(e)) can be used as additional evidences confirming the attachment of propyl groups at the amino moieties of chitosan in the step of reductive *N*-alkylation. For the QBzCS particles (Fig.2(f)), the signals of aromatic protons at $\delta 7.4$ ppm together with the benzylic protons at 1.0 ppm could be used to verify the attachment of benzyl groups at the amino positions of chitosan.

Based on the ^1H NMR analysis, the degree of quaternization (%DQ) can also be estimated. To simplify the calculation, it was assumed that the majority of quaternary ammonium groups existed in the form of $-\text{N}^+(\text{CH}_3)_3$ and only a few were present in the form of $\text{NR}^+(\text{CH}_3)_2$. This assumption is based on the fact that the non-alkylated or free amino groups ($-\text{NH}_2$) left after the *N*-reductive alkylation step are less bulkier than the alkylated amino group ($-\text{NHR}$) and should thus be more accessible for nucleophilic substitution of CH_3I . Such

characteristics should favor the formation of $-N^+(\underline{\text{CH}_3})_3$ as opposed to that of the $\text{NR}^+(\underline{\text{CH}_3})_2$. The %DQ can thus be calculated from the relative ratio between the integration of the nine protons from the three methyl groups of the quaternized *N*-alkyl chitosan at the position δ 3.1-3.3 ppm and the peak integration of the six protons of chitosan (δ 3.3-4.4 ppm). Accordingly, the calculated %DQ value based on the content of $N^+(\underline{\text{CH}_3})_3$ was 2.1, 7.1, 4.2, and 5.7 % for TMCS, QACS, QPCS, and QBzCS particles, respectively. It should be taken into account that the quaternized chitosan particles were not completely soluble in the $\text{CF}_3\text{COOH}/\text{D}_2\text{O}$, the solvent used for NMR analysis. As a result, the %DQ values obtained from ^1H NMR analysis are not capable of reflecting the actual degree of surface quaternization, they can only be used as a qualitative measure of the extent of surface substitution. The fact that %DQ of TMCS particles was the lowest agrees quite well with the data formerly described based on FT-IR analysis (Fig.1).

TEM analysis revealed that all particles were quite spherical in shape and had average diameters in a sub-micron range of 260 -490 nm (Fig. 3). The dimension of QACS particles was quite comparable to that of the TMCS particles implying that the preparation method did not have significant impact on the particle size and morphology. On the other hand, the size of chitosan particles seemed to be greater when being modified by a larger alkyl substituent (propyl, benzyl) which came from the aldehyde used in the reductive *N*-alkylation step. The charge characteristic of the quaternized chitosan particles was determined by photon correlation spectroscopy (PCS). As shown in Fig. 3, all particles prepared by heterogeneous quaternization via reductive *N*-alkylation followed by methylation (QPCS and QBzCS particles) or direct methylation (QACS particles) exhibited greater zeta potential values than those of chitosan and TMC particles. Indeed, comparing TMCS and QACS particles, it seems that the heterogeneous quaternization approach was more efficient in enhancing positive charges of the chitosan particles than the homogeneous one. This may stem from the fact that some positive charges of

TMC were neutralized following ionic crosslinking with the negatively charged TPP upon the particle formation. In accord with this notion is that the TMCS particles formed by the crosslinking of TMC and TPP were not completely soluble in $\text{CF}_3\text{COOH}/\text{D}_2\text{O}$. As a result, the %DQ of TMC particles (2.1%) was much lower than that of the originally synthesized TMC (%DQ = 11.8%) indicating the consumption of the positive charges during crosslinking. Unlike the TMCS particles, the introduction of trimethyl groups to chitosan particles via direct methylation to yield the QACS particles was performed after the particle formation. Thus, the positive charge density generated thereafter was not depleted by the crosslinking. A similar explanation can be applied for the QPCS and QBzCS particles, where again the quaternization was applied after the particle formation. Interestingly, the presence of the larger alkyl substituents (propyl and benzyl) somewhat promoted the subsequent methylation accounting for why the ζ -potential was raised above +35 eV for both the QPCS and QBzCS particles. It is postulated that the more hydrophobic environment created by the propyl and benzyl substituents may increase a chance of CH_3I , a small organic molecule of low polarity, to reach the amino active sites of chitosan chain during methylation. Previous work has also demonstrated that the degree of surface quaternization can be tailored by adjusting the CH_3I concentration used in the methylation step [28].

3.2 Antibacterial efficacy of surface-quaternized chitosan particles

The antibacterial activity of surface-quaternized chitosan particles were tested against the representative Gram-positive and Gram-negative bacteria, *S. aureus* and *E. coli*, respectively, and reported as the total number of replication competent (viable) cells as mean colony forming units per volume (CFU/mL). In the case of *S. aureus*, all quaternized chitosan particles, TMCS, QACS, QPCS and QBzCS particles, exhibited significantly higher antibacterial ratio (> 95%) than the chitosan particles at pH 7.4 (Fig. 4), supporting that the

introduction of the positive charges via quaternization can effectively improve the antibacterial activity of chitosan particles especially in neutral media. In contrast, the antibacterial action of chitosan particles cannot be detected given that their viable counts closely resembled those of the negative control. This outcome is in line with our previous observation on chitosan film implying that chitosan particles would exhibit its potential in suppressing the bacterial growth only when they are in acidic media in which they are positively charged [28]. This also further suggested that the chelation of the amino groups of chitosan as proposed by others [30, 31] is definitely not the major mechanism that controls the antibacterial activity at this particular condition. These evidences support that the superior antibacterial activities of some quaternized chitosan particles, especially QPCS and QBzCS particles, to the chitosan particles are mainly due to their higher charge density in near neutral pH condition than the latter (See Fig. 3 for ζ -potential data). Overall, this trend is in accordance with previously observed data reported by Qi and coworkers [16], who proposed that the positively charged chitosan particles should interact favorably with negatively charged cell membranes, causing an increase in membrane permeability and eventually rupture and leakage of intracellular components. This proposed mechanism is particularly reasonable in the case of the Gram-positive bacteria, such as *S. aureus* since their outer membrane consists of negatively charged teichoic acid, that is believed to be responsible for the electrostatic binding with the positively charged particles.

Although the TMCS particles exhibited a slightly lower ζ -potential than the chitosan particles, they still inhibited the growth of *S. aureus*, potentially suggesting that the inhibition did not solely rely on the contact-inhibitory mechanism which should be charge density-dependent, but may also be influenced by the charge inside the particles or the ability to dissolve in the media considering that TMC is water soluble and not all of the TMC molecules were crosslinked by TPP during the particle formation. In fact, this release-inhibitory

mechanism involving surface leaching of soluble chitosan and/or its quaternized derivatives has been recently proposed by Fernandez-Saiz and co-workers [32] and also in our earlier work on the surface-quaternized chitosan films [28]. Similar explanations may also be applied in the case of QACS particles whose charge density (ζ -potential = +29.3 mV) was slightly higher than those of both chitosan particles (ζ -potential = +27.1 mV) and TMCS particles (ζ -potential = +23.2 mV). Having the same type of quaternary ammonium groups ($-\text{N}(\text{CH}_3)_3$), the QACS particles exhibited better antibacterial action against *S. aureus* than the TMCS particles indicating that the heterogeneous quaternization was potentially a more effective route to promote the antibacterial activity of chitosan particles than the homogeneous one. This is extremely important considering that the higher charge density can be achieved via heterogeneous route without sacrificing the positive charges to the crosslinking process. The lowering of the ζ -potential upon ionotropic crosslinking of TMC by TPP has also been reported by Sadeghi and co-workers [33]. It is also conceivable that the uncrosslinked surface-modified quaternized layer on the surface of QACS particles may leach out easier than the quaternary entities trapped or crosslinked by TPP inside and/or on the surface of TMCS particles.

The trend observed for the representative Gram-negative bacteria, *E. coli* is quite different from that seen with the Gram-positive *S. aureus* described above. In comparison with the negative control and chitosan particles, the quaternized chitosan particles with only slightly higher charge density, namely TMCS, and QACS particles, did not possess significant antibacterial activity against *E. coli* (Fig. 4). QACS particles exhibited % antibacterial activity of 82.9% whereas the detrimental action of TMCS cannot be detected. On the other hand, the quaternized chitosan particles with larger alkyl groups having higher charge density, namely QPCS and QBzCS particles, exerted much higher antibacterial action against the *E. coli*. With a similar ζ -potential, the QBzCS particles displays a greater inhibitory action than the QPCS particles which is reasonable considering that the uppermost

layer of the outer membrane of *E. coli* contains lipopolysaccharide that should interact favorably with hydrophobic entities of the particles. In addition, the fact that QACS film was reported to exhibit much lower % antibacterial activity against *S. aureus* (26% antibacterial ratio) and *E. coli* (29% antibacterial ratio) [28], in comparison with the QACS particles apparently suggests that the antibacterial efficacy can be magnified by the enhanced surface area of the particles.

Comparatively, as outlined in Fig. 4, it was found that *E. coli* had a greater resistance to quaternized chitosan particles than the *S. aureus*. This can be explained by three reasons. Firstly, the absence of an outer membrane and the presence of negatively charged teichoic acid within a thick peptidoglycan layer (20-80 nm) on the surface of *S. aureus* should make them more attractive to the positively charged, quaternized chitosan particles and more specific to be damaged by positively charged molecules than *E. coli*. Secondly, the presence of a number of small channels of porins within the outer membrane of *E. coli* may help block the entrance of the particles into the bacterial cell, making them more difficult to inhibit than *S. aureus*. Finally, the smaller dimension of *S. aureus* (sphere, i.d. $\sim 0.5\text{-}1\text{ }\mu\text{m}$) may partly account for more intimate contact with the quaternized chitosan particles, making the antibacterial activity more effective than the *E. coli* (rod, $0.3\text{-}1.0 \times 1.0\text{-}6.0\text{ }\mu\text{m}$).

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that can inhibit the growth of the target microorganism. From a practical perspective, this value is a key indication of the inhibitory efficiency of the substance against the microorganism. In this study, the MIC values of chitosan, TMCS, QACS, and QBzCS particles were evaluated by viable cell counting methods. The MIC values for each type of particle were identified by comparing the viable cell counts ($\log(\text{CFU/mL})$) between the suspension having the designated particles and the negative control). Results for all quaternized chitosan particles are displayed in Table 1. The fact that the concentration of as

high as 1000 mg/mL was necessary for chitosan particles to express their inhibitory power explained why no inhibitory action was detected when previously tested with 0.5 mg/mL or 500 µg/mL chitosan particles (Fig.4). To start suppressing the growth of *E. coli* by chitosan particles demanded a concentration as high as 2000 µg/mL. These results support the previous assumption that *E. coli* is more tolerant than *S. aureus*. However, using the same growth media for bacteria, MHB, much lower MIC values of 234 and 117 µg/mL against *S. aureus* and *E. coli*, respectively have been reported for the chitosan particles also prepared by ionotropic crosslinking by TPP [24]. It is suspected that this difference may be a result of their particles having a much smaller size (~54 nm) and so possess a greater available surface area than ours (~320 nm). Therefore, a significantly lower quantity of chitosan particles was required in their case. In contrast, Sadeghi and co-workers [33] reported a two-fold higher MIC value for the TPP-crosslinked chitosan particles against *S. aureus*. This is reasonable given that their particles had a comparable size (~250 nm) to ours but with less ζ- potential (+15.9 eV).

As anticipated, the QACS particles with lower MIC were more potent in prohibiting bacterial growth than the TMCS particles. This MIC value (62.5 µg/mL) was apparently lower than those reported for TMC solutions with various degree of quaternization (%DQ) [33, 34] indicating their superior antibacterial efficacy. This outcome truly indicated the potential of heterogeneous route for the preparation of quaternized chitosan particles as opposed to the conventional homogeneous one which is obviously suffered from the drawback of a deteriorated antibacterial activity because the particles have to be formed after quaternization. The data demonstrated by Sadeghi and co-workers (also shown in Table 1) can be used as a good example [33]. The variation in experimental parameters such as molecular weight and degree of deacetylation of chitosan, %DQ, growth media, pH, initial bacteria concentration, bacterial source, method used for determination of the antibacterial

activity (i.e. optical density (OD), total plate counts for viable cells) should be taken into account when comparing the results. Eventhough a greater quantity of QPCS and QBzCS particles was required to start their detrimental effect on both bacterial strains as compared with the QACS particles, their antibacterial action was more effective when the quantity of all particles were as equal to or above the MIC values of the QPCS and QBzCS particles. This can be evidenced from the data shown in Fig.4 (tested with 500 µg/mL particles). Interestingly, the MIC values of both QACS and QBzCS particles were independent on the bacterial strain.

4. Conclusions

Quaternized chitosan particles having different alkyl substitutents can be successfully prepared by heterogeneous direct methylation or reductive *N*-alkylation/methylation as verified by ¹H NMR and FT-IR analyses. The particles were spherical in shape, having an average diameter in a range of 230-490 nm and carried positive charges with ζ-potential ranging from +23.2 to +46.8 mV. As determined by viable count method, it was found that all quaternized chitosan particles exhibited higher antibacterial activity against *S. aureus* (Gram positive bacteria) than the chitosan particles in neutral media. It is believed that the electrostatic binding between the positive charges on the particles and the negatively charged teichoic acid on the outer membrane of *S. aureus* was responsible for their superior antibacterial activity. In contrast, only the QPCS and QBzCS particles, which have a high charge density and relatively large alkyl groups, were capable of suppressing the growth of *E. coli* (Gram-negative bacteria). The fact that *E. coli* had greater resistance to all quaternized chitosan particles than the *S. aureus* may be explained as a result of their difference in shape and outer membrane properties. The minimum inhibitory concentration (MIC) data led to two important conclusive remarks: (1) the antibacterial efficiency of each particle was dose-

dependent and (2) having the same alkyl substituent as TMCS particles, QACS particles were a more potent antibacterial material indicating that the heterogeneous quaternization was a more effective method than the homogeneous one. The results obtained from this research suggested that the surface-quaternized chitosan particles may potentially be used as effective antibacterial fillers for biomedical applications.

Acknowledgments

This research was financially supported by Research Team Promotion Grant from the Thailand Research Fund (RTA50800004). The authors appreciated a language editing service provided by Dr. Robert Butcher, the Publication Counselling Unit (PCU), Faculty of Science, Chulalongkorn University.

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List of Figure and Scheme Captions

Scheme 1 Preparation of surface-quaternized chitosan particles by reductive *N*-alkylation/methylation (top row) and direct methylation (bottom row).

Fig. 1. FT-IR spectra of (a) chitosan particles, (b) TMCS particles, (c) QACS particles, (d) QPCS particles, and (e) QBzCS particles.

Fig. 2. ¹H-NMR spectra of (a) chitosan particles, (b) TMCS particles, (c) QACS particles, (d) QPCS particles, and (f) QBzCS particles.

Fig. 3. Representative TEM micrographs (x 12,000), average particles sizes and ζ- potentials of chitosan and quaternized chitosan particles. Micrographs shown are representative of at least 10 such fields of view per sample and 3 independent samples.

Fig. 4. Total replication competent (viable) cell counts of *S. aureus* and *E. coli* in suspension after incubation with different chitosan particles (0.5 mg/mL) for 24 h. Statistical significance with $p < 0.01$ of the viable count is compared with the negative control (*) and the chitosan particles (#). Below in the table is the corresponding antibacterial ratio.

