NANOPARTICULATE FORMATION USING POLYECTROLYTE COMPLEX BETWEEN CHITOSAN GLUTAMATE AND SHELLAC AS PROTEIN DELIVERY SYSTEM

By

Mr. Pakorn Kraisit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy
Program of Pharmaceutical Technology
Graduate School
Silpakorn University
Academic Year 2012

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การเกิดอนุภาคนาโนโดยการใช้สารโพลิอิเล็กโทรไลโคโทร์ระหว่างไคโตแซนกลูตาแมทและเซลล์ เหล็กเป็นสารประกอบเพื่อใช้ในระบบนำส่งโปรตีน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาบัณฑิตสาขาวิชาเทคโนโลยีเภสัชกรรม นักศึกษา มหาวิทยาลัยศิลปากร ปีการศึกษา 2555

ลิขสิทธิ์ ของบัณฑิตวิทยาลัยมหาวิทยาลัยศิลปากร
The Graduate School, Silpakorn University has approved and accredited the Thesis title of “Nanoparticulate formation using polyelectrolyte complex between chitosan glutamate and shellac as protein delivery system” submitted by Mr. Pakorn Kraisit as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Technology.

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The potential of using two natural polymers (chitosan and shellac) for the formation of nanoparticles by the process of ionic cross-linking to encapsulate bovine serum albumin (BSA), a model protein was investigated. Both polymers were in salt form i.e.; chitosan glutamate (CG) and various hydrolysis times of shellac (HY) in ammonium salt form. Depending on the concentrations of CG, HY and BSA including pH adjustment and various hydrolysis times of shellac, three physical states – nanoparticle, aggregation, and solution could be observed as a result of the electrostatic force. The formation of nanoparticles was due to the balance between the repulsion force and attractive force while the imbalance between both forces resulted in the formation of aggregation and solution. The fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), differential thermal analysis (DTA), X-ray diffraction (XRD) and simultaneous XRD–DSC measurement were applied to prove the nanoparticle formation. The particle size was characterized by the light scattering technique and was found in the range between 100 and 300 nm. The morphology of the particles, detected by transmission electron microscopy (TEM) and scanning electron microscope (SEM) were spherical shape. The result showed that the zeta potential of the nanoparticles possessed positive charges. The concentrations of CG, HY and BSA including pH adjustment and various hydrolysis times of shellac had an influence on the physicochemical properties of the nanoparticles such as the particle size, the zeta potential, the encapsulation, the loading efficiencies and the cumulative release. The enteric coated capsule was used to protect the nanoparticle in SGF pH 2.0 medium and the BSA from the nanoparticles were gradual released in SIF pH 6.8 and 7.4. The MTT assay was used for cytotoxicity test and the various concentrations of sample, pH adjustment, and incubation time had an influence on the cell viability. The uptake of the nanoparticles via the Caco-2 cell was significantly higher than in solution form. The nanoparticles could be kept at least 3 months in dried form at 4°C by using glucose and trehalose as a cryoprotectant. The nanoparticle formation was evaluated for the structure conformation of BSA after freeze drying process and after stability test by using gel electrophoresis, proving that the conformation of BSA did not change in any test conditions. In conclusion, HY could be applied as natural polyanion for polyelectrolyte complex. CG and HY, the natural polymers, are potentially useful polymers for the nanoparticulate carrier as protein and drug delivery systems. Therefore, this nanoparticle can be a potential carrier for further development for oral protein drug delivery system.
สาขาวิชาเทคโนโลยีเภสัชกรรม
บัณฑิตวิทยาlokมหาวิทยาลัยศิลปากร

การศึกษานี้เป็นการศึกษาด้านภาษาในการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พอลิเมอร์จากสารธรรมชาติด้วยการใช้พólิเมอร์จากสารธรรมชาติ ซึ่งคือไคโตแซนและเชลแล็กสำหรับการจู่เป็นเป็นไปในพ่อพืชชีวิตของปัญตะแหงอยู่ในช่วง 100 ถึง 300 ในแบบโดยการวัดด้วยเทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิควิเคราะห์ เ�เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคนิคเเทน์ เทคเ
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>English Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Thai Abstract</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2 Literature Review</td>
<td>5</td>
</tr>
<tr>
<td>3 Materials and Methods</td>
<td>51</td>
</tr>
<tr>
<td>4 Results and Discussion</td>
<td>68</td>
</tr>
<tr>
<td>5 Conclusions</td>
<td>166</td>
</tr>
<tr>
<td>Bibliography</td>
<td>171</td>
</tr>
<tr>
<td>Appendix</td>
<td>195</td>
</tr>
<tr>
<td>Biography</td>
<td>208</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Techniques for oral protein delivery system</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>FDA-approved nanoparticle drug delivery systems in clinical trials and their indications</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Source of chitin and chitosan</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>Principal properties of chitosan in relation to its use in biomedical applications</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>Principal applications for chitosan</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Examples of commonly used excipients in freeze-drying of pharmaceutical products</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>Some of cryoprotectants used for the freeze-drying of nanoparticles</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>Composition of separating gel</td>
<td>62</td>
</tr>
<tr>
<td>9</td>
<td>Composition of stacking gel</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>Concentration of compositions of supplemented EMEM (100 mL)</td>
<td>63</td>
</tr>
<tr>
<td>11</td>
<td>Concentrations of CG or HY and the amount used of HBSS</td>
<td>66</td>
</tr>
<tr>
<td>12</td>
<td>Concentrations of Nanoparticle dispersion and the amount used of HBSS</td>
<td>66</td>
</tr>
<tr>
<td>13</td>
<td>Prepared concentrations of free FITC-BSA and loaded FITC-BSA NP</td>
<td>66</td>
</tr>
<tr>
<td>14</td>
<td>Acid value, % water content and % insoluble solid of HY 0, HY 15, HY 30 and HY 45</td>
<td>73</td>
</tr>
<tr>
<td>15</td>
<td>Water content of HY 0, HY 15, HY 30 and HY 45; before and after freeze drying</td>
<td>74</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Effect of different concentrations of CG, HY 0 and BSA on particle size and zeta potential of BSA-loaded CG-HY NP</td>
<td>84</td>
</tr>
<tr>
<td>17</td>
<td>Effect of different concentrations of CG 35, HY 0 and BSA on Encapsulation efficiency (EE) and Loading efficiency (LE) of BSA-loaded CG-HY NP</td>
<td>92</td>
</tr>
<tr>
<td>18</td>
<td>Effect of different molecular weight of CG and concentrations of BSA on the formation of nanoparticles and aggregation state (HY 30 0.200 %w/v, CG 0.100 %w/v)</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>Effect of different hydrolysis time of shellac and concentrations of BSA on the formation of nanoparticles (HY 0.200 %w/v, CG 35 0.100 %w/v)</td>
<td>104</td>
</tr>
<tr>
<td>20</td>
<td>Effect of different concentrations of CG 35 kDa and BSA on the formation of nanoparticles and aggregation state</td>
<td>107</td>
</tr>
<tr>
<td>21</td>
<td>Effect of different concentrations of HY 30 and BSA on the formation of nanoparticles, solution and aggregation state (CG 35 0.125 %w/v)</td>
<td>112</td>
</tr>
<tr>
<td>22</td>
<td>Effect of pH adjustment of different concentrations BSA on the formation of nanoparticles and aggregation state (CG 35 0.125 %w/v, HY 30 0.200 %w/v)</td>
<td>117</td>
</tr>
<tr>
<td>23</td>
<td>IC₅₀ of samples</td>
<td>157</td>
</tr>
<tr>
<td>24</td>
<td>List of abbreviations</td>
<td>205</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic representation of preparation of chitosan particulate systems by spray drying method.</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Preparation processes of chitosan microspheres by crosslinking method.</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>Ionic gelation of chitosan and TPP.</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Schematic representation of preparation of chitosan particulate systems by ionic cross-linking methods.</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Structures of chitin and chitosan.</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>The positions for modification of chitosan structure.</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>Chemical structure of shellac.</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Lac insect life cycle (Kerria lacca).</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>Stick lac (a), seed lac (b), shellac (c), and button lac (d).</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>Proposed diagram of unplasticized and plasticized shellac film with PEG (200, 400, 4000) after storage at 40 °C, 75% for 3 months</td>
<td>38</td>
</tr>
<tr>
<td>11</td>
<td>Proposed diagram of salt formation of various forms of shellac.</td>
<td>39</td>
</tr>
<tr>
<td>12</td>
<td>Schematic of an intestinal epithelium; The arrows indicate the four different drug transport routes: 1) the passive transcellular, 2) the passive paracellular, 3) the active carrier-mediated transcellular, and 4) the transcytosis routes.</td>
<td>41</td>
</tr>
<tr>
<td>13</td>
<td>Schematic representation of the measurement of contact angle by using sessile drop technique.</td>
<td>57</td>
</tr>
<tr>
<td>14</td>
<td>Physical characteristic of hydrolyzed shellac at various times; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45.</td>
<td>68</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15</td>
<td>SEM photomicrographs of hydrolyzed shellac at various times; (A) native shellac, (B) 15 min, (C) 30 min and (D) 45 min</td>
<td>69</td>
</tr>
<tr>
<td>16</td>
<td>SEM photomicrographs of (A) chitosan base and (B) chitosan glutamate</td>
<td>69</td>
</tr>
<tr>
<td>17</td>
<td>Hot-stage microscopic images of HY 0 at (A) 50 °C, (B) 80 °C, (C) 84°C, (D) 88 °C, (E) 90 °C and (F) 95 °C</td>
<td>71</td>
</tr>
<tr>
<td>18</td>
<td>Hot-stage microscopic images of HY 15 at (A) 50 °C, (B) 70 °C, (C) 75°C, (D) 80°C, (E) 85 °C and (F) 90 °C</td>
<td>71</td>
</tr>
<tr>
<td>19</td>
<td>Hot-stage microscopic images of HY 30 at (A) 50 °C, (B) 65 °C, (C) 70°C, (D) 76°C, (E) 80 °C and (F) 90 °C</td>
<td>72</td>
</tr>
<tr>
<td>20</td>
<td>Hot-stage microscopic images of HY 45 at (A) 50 °C, (B) 65 °C, (C) 60 °C, (D) 65°C, (E) 70 °C and (F) 75 °C</td>
<td>72</td>
</tr>
<tr>
<td>21</td>
<td>Surface characteristic of HY 0, HY 15, HY 30 and HY 45: (A) contact angle, (B) component of surface free energy and (C) total surface free energy</td>
<td>75</td>
</tr>
<tr>
<td>22</td>
<td>DTA thermograms of; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45</td>
<td>76</td>
</tr>
<tr>
<td>23</td>
<td>XRD pattern of; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45</td>
<td>77</td>
</tr>
<tr>
<td>24</td>
<td>FTIR spectra of hydrolyzed shellac at various times; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45</td>
<td>78</td>
</tr>
<tr>
<td>25</td>
<td>XRD-DSC data of HY 0</td>
<td>79</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>26</td>
<td>XRD-DSC data of HY 15</td>
<td>80</td>
</tr>
<tr>
<td>27</td>
<td>XRD-DSC data of HY 30</td>
<td>80</td>
</tr>
<tr>
<td>28</td>
<td>XRD-DSC data of HY 45</td>
<td>81</td>
</tr>
<tr>
<td>29</td>
<td>XRD-DSC data of chitosan base (CH)</td>
<td>82</td>
</tr>
<tr>
<td>30</td>
<td>XRD-DSC data of chitosan glutamate (CG)</td>
<td>82</td>
</tr>
<tr>
<td>31</td>
<td>XRD-DSC data of BSA</td>
<td>83</td>
</tr>
<tr>
<td>32</td>
<td>Three physical states photograph of the ionic interaction: Nanoparticle (A), Aggregation (B) and Solution state (C)</td>
<td>83</td>
</tr>
<tr>
<td>33</td>
<td>Proposed schematic representation of ionic interaction of Nanoparticulate formation (A), Aggregation (B) and solution state (C)</td>
<td>87</td>
</tr>
<tr>
<td>34</td>
<td>TEM photomicrographs of BSA-loaded CG-HY NP (CG 35 0.100 %w/v, HY 0 0.050 %w/v and BSA 1.50 mg/mL)</td>
<td>88</td>
</tr>
<tr>
<td>35</td>
<td>FTIR spectra of (A) HY 0, (B) BSA, (C) CG, (D) BSA-loaded CG-HY NP of CG 35 0.100 %w/v, HY 0 0.050 %w/v and BSA 1.5 mg/mL and (E) CG 35/HY 0/BSA physical mixture 1:1:1</td>
<td>89</td>
</tr>
<tr>
<td>36</td>
<td>DSC thermograms of (A) HY 0, (B) BSA, (C) CG, (D) BSA-loaded CG-HY NP of CG 35 0.100 %w/v, HY 0 0.050 %w/v and BSA 1.5 mg/mL and (E) CG 35/HY 0/BSA physical mixture 1:1:1</td>
<td>91</td>
</tr>
<tr>
<td>37</td>
<td>The release profile of BSA-loaded CG-HY NP at different concentrations of CG 35 and HY 0 in phosphate buffer solution at pH 7.4, 37 °C</td>
<td>93</td>
</tr>
<tr>
<td>38</td>
<td>SEM photographs of BSA-loaded CG-HY NP; of CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL</td>
<td>95</td>
</tr>
</tbody>
</table>
Figure 39 DTA thermograms of (A) HY 30 , (B) CH 35, (C) CG 35, (D) BSA, (E) BSA-loaded CG-HY NP of CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL, and (F) CG 35/ HY 30/BSA physical mixture 1:1:1......................................................... 96

Figure 40 XRD pattern of (A) HY 30 , (B) CH 35, (C) CG 35, (D) BSA, (E) BSA-loaded CG-HY NP of CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL, and (F) CG 35/ HY 30/BSA physical mixture 1:1:1;............................................................... 97

Figure 41 XRD-DSC data of BSA-loaded CG-HY NP of CG 35 0.100 %w/v, HY 30 0.200 %w/v and BSA 1.5mg/mL............................................. 99

Figure 42 Effect of different molecular weight of CG and concentrations of BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (HY 30 0.200 %w/v, CG 0.100 %w/v) 101

Figure 43 Effect of different molecular weight of CG and concentrations of BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (HY 30 0.200 %w/v, CG 0.100 %w/v).......................................................... 103

Figure 44 Effect of different hydrolysis time of shellac and concentrations of BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (HY 0.200 %w/v, CG 35 0.100 %w/v)... 105

Figure 45 Effect of different hydrolysis time of shellac and concentrations of BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (HY 0.200 %w/v, CG35 0.100 %w/v)........................................ 106

Figure 46 Effect of different concentrations of CG 35 and BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (HY 30 0.200 %w/v)......................................................... 109
47 Effect of different concentrations of CG 35 and BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (HY 30 0.200 %w/v)........... 111

48 Effect of different concentrations of HY 30 and BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (CG 35 0.125 %w/v)........................................... 113

49 Effect of different concentrations of HY 30 and BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (CG 35 0.125 %w/v)........... 115

50 Effect of pH adjustment of different concentrations BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (CG 35 0.125 %w/v, HY 30 0.200 %w/v).................. 118

51 Effect of pH adjustment of different concentrations BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (CG 35 0.125 %w/v, HY 30 0.200 %w/v).................................................. 120

52 The release profile of BSA-loaded CG-HY_HY NP in SGF pH 2.0, 37 °C, (CG 35 0.125 %w/v, HY 30 0.200 %w/v).................. 123

53 The release profile of BSA-loaded CG-HY_HY NP at different molecular weights of CG in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL)................................................................. 123

54 The release profile of BSA-loaded CG-HY_HY NP at different hydrolysis time of shellac in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (CG 35 0.125 %w/v, HY 0.200 %w/v and BSA 1.5 mg/mL)................................................................. 124
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>The release profile of BSA-loaded CG-HY_HY NP at different concentrations of CG 35 in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37°C, (HY 30 0.200 %w/v, BSA 1.5 mg/mL)</td>
<td>124</td>
</tr>
<tr>
<td>56</td>
<td>The release profile of BSA-loaded CG-HY_HY NP at different concentrations of HY 30 in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37°C, (CG 35 0.125 %w/v, BSA 1.5 mg/mL)</td>
<td>125</td>
</tr>
<tr>
<td>57</td>
<td>The release profile of BSA-loaded CG-HY_HY NP at different concentrations of BSA in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37°C, (CG 35 0.125 %w/v, HY 30 0.200 %w/v)</td>
<td>125</td>
</tr>
<tr>
<td>58</td>
<td>Effect of stability of BSA-loaded CG-HY_HY NP at kept in colloidal form at 4 °C on (A) pH, (B) zeta potential and (C) particle size (CG 35 0.125 %w/v, HY 30 0.200 %w/v, and Glu 7.5 %w/v)</td>
<td>127</td>
</tr>
<tr>
<td>59</td>
<td>Effect of Glu on stability of BSA-loaded CG-HY_HY NP (0.125%w/v CG 35, 0.200 % w/v HY 30, and Glu 7.5 %w/v) in colloidal form at 4 °C : (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (CG 35 0.125 %w/v, HY 30 0.200 %w/v, and Glu 7.5 %w/v)</td>
<td>128</td>
</tr>
<tr>
<td>60</td>
<td>Physical photograph of the nanoparticle; before freeze drying (A), and after freeze drying (B) (using Glu as a cryoprotectant)</td>
<td>129</td>
</tr>
<tr>
<td>61</td>
<td>Effect of different concentrations of CG 35 on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) pH, (B) zeta potential and (C) particle size ( HY 30 0.200 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)</td>
<td>130</td>
</tr>
<tr>
<td>Figure</td>
<td>Effect of different concentrations of CG 35 on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE)</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>(HY 30 0.200 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)………………………………………………………………………………………………………………………… 132</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>Effect of different concentrations of HY 30 on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C (A) pH, (B) zeta potential and (C) particle size (CG 35 0.125 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)…………………………………… 134</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>Effect of different concentrations of HY 30 on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (CG 35 0.125 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)…………………………………… 136</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Effect of different hydrolysis time of shellac on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) pH, (B) zeta potential and (C) particle size (CG 35 0.125 %w/v, HY 0.200 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)…………………………………… 138</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>Effect of different hydrolysis time of shellac on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (CG 35 0.125 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)…………………………………… 140</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>Effect of different concentrations of BSA on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) pH, (B) zeta potential and (C) particle size (CG 35 0.125 %w/v, HY 30 0.200 %w/v and Glu 7.5 %w/v)…………………………………………………… 142</td>
<td></td>
</tr>
</tbody>
</table>

xvi
Figure 68: Effect of different concentrations of BSA on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (CG 35 0.125, HY 30 0.200 %w/v and Glu 7.5 %w/v). Page: 144

Figure 69: Effect of different concentrations of glucose and trehalose on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) pH, (B) zeta potential and (C) particle size (CG 35 0.125, HY 30 0.200 %w/v and BSA 1.5 mg/mL). Page: 146

Figure 70: Effect of different concentrations of Glu and Tre on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL). Page: 148

Figure 71: Effect of different concentrations and molecular weights of CG on relative cell viability incubated for 2 h, pH of sample adjusted at 6.0 (A) and 7.4 (B). Page: 151

Figure 72: Effect of different concentrations and molecular weights of CG on relative cell viability incubated for 24 h, pH of sample adjusted at 6.0 (A) and 7.4 (B). Page: 152

Figure 73: Effect of different concentrations of hydrolyzed shellac and hydrolysis time on relative cell viability incubated for 2 h, pH of sample adjusted at 6.0 (A) and 7.4 (B). Page: 154

Figure 74: Effect of different concentrations of hydrolyzed shellac and hydrolysis time on relative cell viability, pH of sample adjusted at 7.4 and incubated for 24 h. Page: 155
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>156</td>
</tr>
<tr>
<td>76</td>
<td>158</td>
</tr>
<tr>
<td>77</td>
<td>159</td>
</tr>
<tr>
<td>78</td>
<td>160</td>
</tr>
<tr>
<td>79</td>
<td>162</td>
</tr>
<tr>
<td>80</td>
<td>163</td>
</tr>
<tr>
<td>81</td>
<td>164</td>
</tr>
<tr>
<td>82</td>
<td>165</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>83</td>
<td>Standard curve of BSA solution for encapsulation and loading efficiency study</td>
</tr>
<tr>
<td>84</td>
<td>Schematic of capsule before (A), and after coating with methacrylic acid and methyl methacrylate (B)</td>
</tr>
<tr>
<td>85</td>
<td>The effect of HY 0 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>86</td>
<td>The effect of HY 15 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>87</td>
<td>The effect of HY 30 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>88</td>
<td>The effect of HY 45 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>89</td>
<td>The effect of CG 35 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>90</td>
<td>The effect of CG 45 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>91</td>
<td>The effect of CG 200 on cytotoxicity incubated with Caco-2 cells</td>
</tr>
<tr>
<td>92</td>
<td>Standard curve of FITC-BSA solution for cellular uptake study</td>
</tr>
<tr>
<td>93</td>
<td>Standard curve of FITC-BSA NP for cellular uptake study</td>
</tr>
<tr>
<td>94</td>
<td>Standard curve of BSA solution for protein content in cellular uptake study</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Nowadays, protein and peptide drug delivery have greatly been used for treatment in several disease conditions. Many researchers have attempted to investigate for new routes or methods to deliver protein and peptide drug to the body. The parenteral route is the major delivery of these therapeutic peptides due to the higher effective than other routes. Most of these therapeutic peptides are still administered by the parenteral route because of their poor bioavailability when delivered via other routes. Generally, peptide drugs are used for chronic conditions, and the use of injections on a daily basis during long-term treatment [1]. The widely studied therapeutic peptide is insulin. The subcutaneous is the major route of insulin delivery. The problems via this route are the burden of daily injections, physiological stress, pain, inconvenience, cost, risks, infection, instability to handle, and the localized deposition of insulin, leading to local hypertrophy and fat deposition at the injection sites [2]. Consequently, the results of research into several aspects of the delivery of the insulin and another protein drugs are available. In recent years, other routes apart from parenteral such as oral, nasal, buccal, pulmonary, transdermal, rectal, and ocular have been a great deal of interest in the exploitation of non-invasive routes for insulin or another protein drugs delivery, and their development by the pharmaceutical industry [3].

Oral administration is the route of choice for the administration of most drugs, regardless of their molecular structure or weight. In production process of an oral dosage form does not have to meet specialized regulatory requirements such as sterility, pyrogenicity, and particulate contamination [4]. However, the physical and chemical instabilities of proteins are the main problems in the development of oral protein drug delivery. This is a reason of the traditional preference route to oral [5]. Problems of oral protein drug delivery such as acid catalyzed degradation, proteolytic enzyme, poor permeability across the gastrointestinal mucosa and first-pass metabolism during transfer across the absorption barrier and in the liver have been
challenged for the efficient delivery of oral protein drugs into the blood stream [6]. The successful delivery of oral protein drugs through the GI tract is to protect them from the harsh environment in the stomach [7]. The use of nanocarriers for entrapping oral protein drug is a possible choice to overcome these barriers by preventing the enzymatic attack and crossing through the mucosal barrier in GI tract. The particle size and the character of surface of these nanocarriers are important factors for passing mucosa [8]. Additionally, the efficacy for nanocarrier could be improved by using mucoadhesive and absorption enhancing polymers [9].

Nanoparticulate delivery system has been greatly interested, and widely investigated in pharmaceutical industry due to an ability of control release of peptide drugs, a protection from degradation in the GI tract and an enhancement of transmucosal transport enabling to an improvement of bioavailability [10, 11]. A macromolecule or a particle can theoretically cross the intestinal epithelium by the paracellular route (between adjacent cells) and the transcellular route, the transcellular route being the most explored [12]. Several techniques have been used for the preparation of nanoparticles for oral delivery system such as the solvent evaporation, the interfacial polymerization and the emulsion polymerization methods. These techniques involve heat, organic solvent and violent agitation processes which are complicated to execute and can be potentially harmful to therapeutic proteins and peptides [11]. Ionic cross-linking is a popular technique due to its simplicity and mild condition. The technique involves the cross-linking between cationic molecules and anionic molecules via electrostatic interaction [13, 14]. The most commonly used cationic polymer is chitosan and the anionic material is tripolyphosphate (TPP).

Chitosan, a cationic natural biopolymer, produced from deacetylation of chitin [poly-β-(1-4)-N-acetyl-D-glucosamine] which can be extracted from crustaceans, insects, fungi, etc [15-19]. The advantages of using chitosan is biocompatible, biodegradable, low toxicity, good mucoadhesion and membrane permeable enhancing properties by opening of tight epithelial cell junctions [18, 20, 21]. It is soluble in an acidic solution at pH below 6.4-6.5 [22, 23]. In addition, the protonation of primary amino groups at lower pH contributes to the positive charges for cross-linking with polyanions especially tripolyphosphate (TPP) to form nanoparticles for drug and protein delivery system [24-26]. Several attempts have
been made to employ biomaterials possessing polyanions, such as alginate and pectin to replace tripolyohosphate in the formation of nanoparticulate polyelectrolyte complexes [7, 19]. The polyelectrolyte complexes were formed by the electrostatic interactions between cationic and anionic polymers to act as the carrier for drug, peptide and protein and gene delivery systems [7, 19, 27]. In this study, the natural biopolymer, shellac possessing polyanion instead of TPP was explored. Shellac produced from lac insect *Laccifer Laccia* is abundant in Thailand, China and India. The resinous secretion can be purified to yield shellac which has been used in the food industry, paint industry and, in a lesser extent, in the pharmaceutical industry [28, 29]. Shellac begins to dissolve above pH 7.0 (the pKa of shellac is 6.9-7.5) which can provide an advantage to be used in the pH of the body fluid (pH 7.4) [30]. The main structure of shellac is the combination of polyesters and single esters consisting of hydroxyl and carboxyl groups [28]. Therefore, shellac possesses the ability to deprotonate when it dissolves in alkaline solution and displays polyanions enabling the formation of polyelectrolyte complex with chitosan. Limmatvapirat et al. [30] modify shellac by partial hydrolysis with 2.0% (w/w) NaOH for different times. The acid value (AV) of shellac increased with prolongation of hydrolysis time. The results suggested that the ester bonds are broken down to free carboxyl group during alkali treatment. With the increased amount of free carboxyl group of shellac by partial hydrolysis, the solubility of shellac was enhanced nearby to pH 7.0 and the hydrolyzed shellac obtained was more effective for ammonium salt formation in ammonia-based film coating [30].

The purposes of this study were to; (I) prepare nanoparticles from two natural polymer (chitosan, acationic and shellac, an anionic) for the formation of nanoparticles by the process of ionic cross-linking to encapsulate bovine serum albumin (BSA); (II) prepare nanoparticle from hydrolyzed shellac and chitosan glutamate for the formation of nanoparticles by the process of ionic cross-linking to encapsulate BSA; (III) evaluate permeation of nanoparticles and viability of cell by using in-vitro cell culture method.

The hypothesis to be tested of this study were to; (I) the possible of using two natural polymers (chitosan, a cationic and shellac, an anionic) for the formation of nanoparticles by the process of ionic cross-linking to encapsulate BSA; (II) the
increase in polyanion of shellac by hydrolysis process could be easier to form complex with chitosan glutamate to encapsulate BSA; (III) the nanoparticle could be greater permeated in vitro cell than solution form.
CHAPTER 2
LITERATURE REVIEW

1. Technique for oral protein delivery system

Various challenges are usually evaluated by determining the result of protein in the GIT. There are many attempts to overcome the enzymatic degradation and the permeation of protein through the GIT [4]. The research about oral protein delivery system is shown in Table 1. Several techniques to increase bioavailability of protein are described as the following. They are absorption enhancers, enzyme inhibitors, mucoadhesive polymeric system, particulate carrier delivery systems, and targeted delivery systems.

1.1 Absorption enhancers

Absorption enhancers improve the absorption of drugs by increasing their paracellular and transcellular transport. They involve several different mechanisms of action, including changes in membrane fluidity, decrease in mucus viscosity, the leakage of proteins through membranes, and the opening of tight junctions [1]. For examples of non-specific permeation enhancers are bile salts, fatty acids, surfactants, salicylates, chelators, and zonula occludens toxin [4]. A study of N-lauryl-β-D-maltopyranoside indicated that this enhancer may open the tight junctions of the intestine epithelium, so the permeation of insulin through a paracellular pathway is reported [31]. Some reports showed the hypoglycemic effects of enteric-coated capsules containing insulin formulated in Witepsol W35 with sodium salicylate, which significantly decreased plasma glucose levels and increased hypoglycemia relative to the effects of a subcutaneous injection of regular soluble insulin [32]. In diabetic rats, the bioavailability of oral insulin co-administered with zonula occludens toxin was sufficient to lower serum glucose concentrations to levels similar to parenteral insulin after injection [33]. However, the use of absorption enhancers is limited by the surface area and amounts of tight junctions on the epithelium. Additionally, transport is enhanced not only for peptide and protein drugs
but also for undesirable molecules present in the GIT [4]. However, an incorporation of so-called permeation enhancers into protein formulations has been shown to increase the permeability of the mucosal epithelium. The permeability enhancement has been reported following application of excipients with varied properties which act by different mechanisms, including increasing membrane fluidity (e.g. surfactants), decreasing mucus viscosity (mucolytic agents), disrupting tight junctions (chitosan, calcium chelators such as EDTA, peptides) [34]. However the exact mechanism to enhance absorption by using some permeability enhancers is unclear [35].
Table 1  Technique for oral protein delivery system

<table>
<thead>
<tr>
<th>System</th>
<th>Application</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption enhancers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile salt/fatty acid mixed micellar system</td>
<td>In vivo/rats</td>
<td>Improved paracellular absorption</td>
</tr>
<tr>
<td>Insulin solution/N-lauryl-N-o-maltopyranoside</td>
<td>In vitro/Using chamber</td>
<td>Enhanced colon permeability</td>
</tr>
<tr>
<td>W/O/W emulsion/DHA or EPA</td>
<td>In situ/rat intestine</td>
<td>PAₐ was 42.2%</td>
</tr>
<tr>
<td>Enteric-coated capsule/White susp W35- NaSal</td>
<td>In vivo/dogs</td>
<td>PAₐ was 12.6%</td>
</tr>
<tr>
<td>Insulin solution/AP, SGC, SC or NaEDTA</td>
<td>In situ/rat intestine</td>
<td>PAₐ was 0.1%–5.5%</td>
</tr>
<tr>
<td>Enzyme inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug–carrier matrix/BBI and elastatin</td>
<td>In vitro/artificial intestinal fluid</td>
<td>Significant reduction in enzymatic degradation</td>
</tr>
<tr>
<td>Insulin solution/AP, SGC, STI, CM or BAC</td>
<td>In situ/rat intestine</td>
<td>PAₐ was 0.1%–5.1%</td>
</tr>
<tr>
<td>Insulin solution/SGC, BTT, LPT, CTT or BAC</td>
<td>In situ/rat intestine</td>
<td>PAₐ was 0.1%–2.3%</td>
</tr>
<tr>
<td>Insulin solution/ClOVM or DlOVM</td>
<td>In vitro/diffusion chamber</td>
<td>Two fold increase in insulin stability and flux values</td>
</tr>
<tr>
<td>Insulin solution/hyaluronidase</td>
<td>In situ/rat intestine</td>
<td>Significant reduction in blood glucose levels</td>
</tr>
<tr>
<td>Macromolecular polymeric systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(MAA-g-EG) hydrogel microspheres</td>
<td>In vivo/rats</td>
<td>PAₐ was 9.5%</td>
</tr>
<tr>
<td>Lectin-conjugated alginate microspheres</td>
<td>In vivo/rats</td>
<td>Hypoglycemic effect lasted for 8 h</td>
</tr>
<tr>
<td>Chitosan NPs</td>
<td>In vivo/rats</td>
<td>PAₐ was 14.9%</td>
</tr>
<tr>
<td>Chitosan–TBA–insulin tablets</td>
<td>In vivo/rats</td>
<td>PAₐ was 1.69%</td>
</tr>
<tr>
<td>Particulate carrier delivery systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin-based micromulsion</td>
<td>In vivo/rats</td>
<td>30% reduction in blood glucose levels</td>
</tr>
<tr>
<td>Double liposomes</td>
<td>In vivo/rats</td>
<td>PAₐ was 0.39%–5.5%</td>
</tr>
<tr>
<td>Fusogenic liposomes</td>
<td>In situ/rat intestine</td>
<td>PAₐ was 10.1%–15.7%</td>
</tr>
<tr>
<td>Extralipid S100 microspheres</td>
<td>In vivo/rabbits</td>
<td>24% reduction in blood glucose levels</td>
</tr>
<tr>
<td>Insulin–phospholipid complex NPs</td>
<td>In vivo/rats</td>
<td>PAₐ was 7.7%</td>
</tr>
<tr>
<td>Targeted delivery systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color-targeted delivery system (CODEST™)</td>
<td>In vivo/dogs</td>
<td>BA was 0.5%</td>
</tr>
<tr>
<td>Color-targeted delivery system (Azopolymer-coated pellets)</td>
<td>In vivo/rats</td>
<td>PAₐ was 0.89%–3.38%</td>
</tr>
<tr>
<td>Insulin–transferrin conjugate</td>
<td>In vivo/rats</td>
<td>70% reduction in blood glucose levels</td>
</tr>
</tbody>
</table>

1.2 Enzyme inhibitors

Recent studies have evaluated the use of enzyme inhibitors to decrease the rate of protein degradation. The co-administration of enzyme inhibitors could help peptide and protein drugs to avoid the enzymatic barrier in the GIT. For example of protein, insulin is strongly degraded by trypsin, α-chymotrypsin, and elastase, and to a lesser extent, by brush-border membrane-bound enzymes [36]. From the report of Yamamoto et al. evaluated the effects of five different enzyme inhibitors—sodium glycocholate, camostat mesilate, bacitracin, soybean trypsin inhibitor, and aprotinin—on the intestinal metabolism of insulin in rats [37]. Among these enzyme inhibitors, sodium glycocholate, camostat mesilate, and bacitracin are effective in improving the physiological availability of protein in the large intestine. However, none of these enzyme inhibitors is effective in the small intestine due to the numerous enzymes secreted there. Liu et al. also evaluated the potential utility of various enzyme inhibitors in improving the intestinal absorption of protein and investigated their efficacy in different intestinal regions [38]. However, the use of enzyme inhibitors may also affect the absorption of other peptides or proteins that would normally be degraded. A major drawback of these inhibitors is their high toxicity, especially for chronic drug therapy. Furthermore, the non-site-specific intestinal application of such compounds may change the metabolic pattern in the GIT, because of the reduced digestion of food proteins.

1.3 Mucoadhesive polymeric systems

The term ‘mucoadhesion’ refers to the adhesion between polymeric carriers and the mucosa and is exhibited by certain polymers, which become adhesive upon hydration [39]. Thus, the goals of mucoadhesive drug delivery systems are to extend the residence time at the site of drug absorption, to increase contact with the mucus, to increase the drug concentration gradient, to ensure immediate absorption without dilution or degradation in the luminal fluid, and to localize the drug delivery system to a certain site [40, 41]. In the 1980s it was believed that (I) strong hydrogen-bonding groups (-OH, -COOH), (II) strong anionic charges, (III) high molecular weight and (IV) sufficient chain flexibility are responsible for mucoadhesion. However, at the beginning 1990s, the contrast of this theory was reported by Hassan and Gallo (1990). They could demonstrate that the positively charged polymer
chitosan also displays strong mucoadhesive properties. These phenomena can be explained by electrostatic interactions of the polymer with negatively charged groups such as sialic acid moieties of the mucus layer [42, 43]. Delivery systems containing mucoadhesive polymers provide close contact with the mucosa, therefore reducing drug degradation between the delivery system and the absorbing membrane. Novel polymers have shown excellent inhibitory activity against proteolytic enzymes and reasonable mucoadhesivity, and hence they might be a useful tool in overcoming the enzymatic barrier to oral peptide therapeutics. The binding of hydrophilic polymers, such as polyacrylates, cellulose derivatives, and chitosan derivatives, to biological surfaces is based on hydrogen bonding and ionic interactions. In the last few years, a large number of mucoadhesive systems have been developed, including superporous hydrogel-composite-based systems [44], lipid-based nanocarriers [45], thiolated polymers [46], and chitosan-based carriers [47, 48]. Some mucoadhesive polymers have also been shown to act as absorption enhancers or inhibitors of proteolytic enzymes [49].

1.4 Targeted delivery systems

The problem of absorption is not uniform throughout the GIT due to the differences in the composition and thickness of the mucus layer, pH, surface area, and enzyme activity [50]. Therefore, the desire to deliver protein and peptide biopharmaceuticals has been made through specific routes of administration which is a challenge. The main purpose of delivery of proteins and peptides to specific sites of action has been used to lower the total dose of them and to concentrate the therapeutic dose at specific sites of pharmacological action [51]. The delivery to the colon has several attractive aspects, including a prolonged residence time, reduced enzymatic activity, increased tissue responsiveness to absorption enhancers, and natural absorptive characteristics [52]. However, the harsh hydrolytic environment of the GIT and the epithelial barriers to absorption are the major challenges to the success of this mode of drug delivery for peptide and protein drugs.

1.5 Particulate carrier delivery systems

Most oral delivery strategies for protein based on particulate carriers individually in nanoparticulate size have been developed to avoid the barriers to oral peptide delivery. Generally, nanometric carriers also comprise sub-micro particles
with size below 1000 nm and with various morphologies, including nanospheres, nanocapsules, nanomicelles, nanoliposomes, and nanodrugs, etc. [53, 54]. Nanoparticles have found widespread use in various routes in drug delivery, counting more than a dozen FDA-approved variants with indications ranging from cancer to infection (Table 2). Nanoparticle drug delivery systems have outstanding advantages [1]: (I) they can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged; (II) they can penetrate through cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord and lymph; (III) they could show controlled-release properties due to the biodegradability, pH, ion and/or temperature sensibility of materials; (IV) they can improve the utility of drugs and reduce toxic side effects; etc. Currently, the researches on nanoparticle drug delivery system focus on: (I) the selectness and combination of carrier materials to obtain suitable drug release speed; (II) the surface modification of nanoparticles to improve their targeting ability; (III) the optimization of the preparation of nanoparticles to increase their drug delivery capability, their application in clinics and the possibility of industrial production; (IV) the investigation of in vivo dynamic process to disclose the interaction of nanoparticles with blood and targeting tissues and organs, etc [55]. They efficiently protect protein and peptide drugs of nanoparticle against enzymatic degradation in the harsh environment of the GIT, provide high transfer of drugs across the epithelial mucosa, control the release rate, and target drug delivery to specific intestinal sites [10, 11, 56]. The pancreatic serine-proteases: trypsin, chymotrypsin and elastase are in many cases responsible for the pre-systemic metabolism of per-orally given (poly) peptide drugs. Ikesue et al. [57], for instance, demonstrated that insulin is strongly degraded by trypsin, chymotrypsin and elastase, whereas almost no degradation caused by brush border membrane bound enzymes could be observed. The pre-systemic metabolism of per-orally peptide drugs is in many cases due to the pancreatic serine-proteases such as trypsin, chymotrypsin and elastase. Strategies to avoid such a pre-systemic metabolism include the use of liposomes, micro- and nanoparticles protecting the incorporated peptide drug towards an enzymatic attack in the gut [16]. For oral absorption, a macromolecule or a particle can theoretically cross the intestinal epithelium by the paracellular route (between adjacent cells) and the
transcellular route, the transcellular route being the most explored [12]. In particular for peptides displaying a molecular size greater than 30 Å, the intestinal membrane becomes an important rate limiting factor for drug absorption [16]. Nanoparticles administered orally can be absorbed by the numerous gut enterocytes such as the membranous epithelial cells (M cells) of Peyer's patches in the gut-associated lymphoid tissue (GALT). Peyer's patches are follicles of lymphoid tissue covered with a specialized epithelium containing M cells. Particle transport by M cells is predominantly transcellular and energy-dependent [58]. Uptake of particles, microorganisms and macromolecules by M cells occurs through fluid phase endocytosis, adsorptive endocytosis and phagocytosis [59]. Factors influencing nanoparticles uptake by M cells are nanoparticle size, hydrophobicity/hydrophilicity balance and the presence of a targeting molecule at the nanoparticle surface [12]. It is generally accepted that nanoparticles below 1 μm are taken up by M cells and delivered in the basal medium, while particles larger than 5 μm are taken up by M cells but remain entrapped in Peyer's patches [60]. However, the optimal size for a nanoparticle to be transcytosed by M cell would be below 1 μm [61, 62] and more precisely below 200 nm [63, 64]. Particle interaction with M cell surface can also rely on the nanoparticle surface charge (zeta potential). Indeed, charged nanoparticles are taken up, but it was a lower extent than non-ionic hydrophobic nanoparticles. Shakweh et al. [65] described that nanoparticles of negative or neutral zeta potential were better transported by Peyer's patches compared with positively charged nanoparticles. Jung et al. reported that the best combination of a hydrophobic and negatively charged nanoparticle would be suitably absorbed through M cells [53]. In physiological conditions, the paracellular route is limited due to the very small surface area of the intercellular spaces and by the tightness of the junctions between the epithelial cells (pore diameter between 3 and 10 Å) [12]. The mucosal epithelial cells are connected by tight junctions, with paracellular space in order of 10 Å and 30–50 Å. This suggests that a ‘particle’ with a radius greater than 15 Å (approximately 3.5 kDa) cannot be transported via this route [34, 66]. The extent of absorption of submicron particles varies in different studies, and is dependent on the nature of the particles employed, their surface charge, their colloidal stability, the dose given and the species of animal [67]. Consequently, a delivery of large peptides and proteins
across mucosal epithelia using paracellular pathway is severely restricted. However, an incorporation permeation enhancers into protein formulations has been shown to increase the permeability of the mucosal epithelium [34]. Free insulin did not affect glycemia when administered orally under the same experimental conditions. In fed diabetic rats, 100 U/kg of encapsulated insulin were able to reduce glycemia by 25% for 6 days after oral administration. Thus, insulin associated with nanocapsules induced a sustained reduction in glycemia when it was given orally to diabetic rats, but efficacy was more obvious in fasted than in fed diabetic animals [68]. Thus, the strategy which utilizes a promoter of absorption or a protease inhibitor in association with micro- or nanoparticulates may be useful for enhancing the efficacy of oral protein formulations. Several techniques have been used for the preparation of nanoparticles for oral delivery system such as the chemical cross-linking methods, drying techniques and the ionic cross-linking methods.

Table 2  FDA-approved nanoparticle drug delivery systems in clinical trials and their indications

<table>
<thead>
<tr>
<th>Therapeutic agent (trade name)</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomal amphotericin B (Ambisome, Ablecet, Amphotech)</td>
<td>Fungal infections, Leishmaniasis</td>
</tr>
<tr>
<td>PEG-adenosine deaminase (Pegadamine)</td>
<td>Severe combined immunodeficiency disease</td>
</tr>
<tr>
<td>PEG-stabilized liposomal doxorubicin (Doxil, Evacet)</td>
<td>Kaposi's sarcoma, refractory ovarian cancer</td>
</tr>
<tr>
<td>Liposomal cytosine arabinoside (DepoCyt)</td>
<td>Lymphomatous meningitis, neoplastic meningitis</td>
</tr>
<tr>
<td>Interleukin 2-diphtheria toxin fusion protein (Denileukin Diftotox)</td>
<td>Cutaneous T-cell lymphoma</td>
</tr>
<tr>
<td>Liposomal verteporfin (Visudyne)</td>
<td>Wet macular degeneration</td>
</tr>
<tr>
<td>PEG-interferon α-2b (Pegasys)</td>
<td>Hepatitis C</td>
</tr>
<tr>
<td>PEG-granulocyte colony stimulating factor (Neulasta)</td>
<td>Chemotherapy associated neutropenia</td>
</tr>
<tr>
<td>Protein bound paclitaxel (Abraxane)</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>PEG-l-asparaginase ( Oncaspar)</td>
<td>Acute lymphocytic leukemia</td>
</tr>
<tr>
<td>PEG aptinib (Macugen)</td>
<td>Wet macular degeneration</td>
</tr>
<tr>
<td>Pemetrexed (Alimta)</td>
<td>Malignant pleural mesothelioma</td>
</tr>
</tbody>
</table>

2. Techniques for the preparation of micro/nanoparticle formulations

Various techniques are used for preparation of micro or nanoparticles such as the solvent evaporation, the interfacial polymerization, the emulsion polymerization, drying techniques, chemical cross-linking method and ionic cross-linking method. It has many factors which are concerned to the micro/nanoparticle formulation process such as material, step of processing, solution or organic solvent used. In this review, we emphasize only the process of drying techniques, chemical cross-linking method and ionic cross-linking method due to the simplicity in preparation. Chitosan is the studied polymer because of many great properties such as biodegradable, biocompatible, mucoadhesive property and, it is popular to use in several researches individually in drug or protein delivery system.

2.1 Drying techniques used for the preparation of protein-loaded chitosan-based particles

Different drying processes have been recently used for preparation of chitosan–protein powder formulations [69-73]. Spray-drying is a well-known technique to produce powders, granules or agglomerates from the mixture of drug and excipient solutions as well as suspensions. The method is based on drying of atomized droplets in a stream of hot air. In this method, chitosan is first dissolved in aqueous acid solution, drug is then dissolved or dispersed in the solution and then, a suitable cross-linking agent is added. This solution or dispersion is then atomized in a stream of hot air. Atomization leads to the formation of small droplets, from which solvent evaporates instantaneously leading to the formation of free flowing particles [74] as depicted in Figure 1. Various process parameters are controlled to get the suitable size of particles. Particle size depends upon the size of nozzle, spray flow rate, atomization pressure, inlet air temperature and extent of cross-linking [13]. The study of mannitol microspheres containing protein-loaded chitosan nanoparticles suitable for pulmonary delivery were prepared by spray drying of a chitosan–FITC-BSA nanoparticles suspension in an aqueous mannitol solution [75]. Mannitol stabilizes the protein structure and improves aerosolization of protein drugs into lungs. FITC-BSA loaded chitosan nanoparticles (300–400 nm) were homogenously encapsulated in mannitol microparticles with a mean diameter of 2.7 μm, adequate for pulmonary delivery.
However, the structural integrity of the encapsulated protein of these particles was not reported [75].

Supercritical fluid (SCF) drying has been recently investigated as an alternative process for producing powder formulations [76-78]. SCF drying is a fast, mild process and produced small microparticles but it has a highly effective cost [76-78]. The most widely used SCF for pharmaceutical applications is carbon dioxide (CO2) because it has a low critical temperature (31.2 °C) and pressure (75.8 bar), and it is non-flammable, non-toxic and inexpensive [78]. Because proteins have a very low solubility in supercritical CO2 (SC-CO2), this fluid has been used as an antisolvent to precipitate proteins from their aqueous solutions [79].

Figure 1  Schematic representation of preparation of chitosan particulate systems by spray drying method
2.2 Chemical cross-linking methods

Chitosan-based particles can be formed by chemical processes, e.g. by the reacting of the primary amine groups of chitosan with a di-aldehyde (mostly glutaraldehyde) cross-linker. For example, a water-in-oil (w/o) emulsion of chitosan with the drug in a water-immiscible solvent (e.g. liquid paraffin) is formed, after which glutaraldehyde is added to crosslink chitosan to yield drug-loaded microspheres [80]. In another study, insulin-loaded chitosan microspheres were prepared by dissolving the protein and the polymer in an acetic acid solution. This solution was subsequently emulsified in mineral oil and chitosan was chemically crosslinked with ascorbyl palmitate or dehydroascorbyl palmitate. This preparation method, high loading of insulin in the microparticles was shown, and they completely released the drug in an active form in about 80 h at an almost constant release rate [81]. As the same study, mineral oil was used as oil phase to make a w/o emulsion. Ascorbyl or dehydroascorbyl palmitate, as interfacial crosslinkers, was used for protecting the protein from the high interfacial surface tension. However, the authors did not discuss about a possible chemical modification of the proteins by the crosslinking agents [81].

Wang et al. prepared uniform-sized protein-loaded chitosan microspheres by a step-wise crosslinking [82]. First, a w/o emulsion of chitosan/insulin in paraffin/petroleum ether mixture was prepared and extruded through a membrane with uniform pores. Then, tripolyphosphate (TPP) was added as an ionic crosslinker followed by the addition of glutaraldehyde to stabilize the microspheres as shown in Figure 2. In another study, monodisperse microspheres of chitosan and/or a quaternized chitosan derivative, N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC), were prepared by a one-step or a two-step chemical crosslinking method using p-phthaldehyde and/or glutaraldehyde as crosslinker. BSA was post-loaded into the formed microspheres [83]. Chitosan-based particles loaded with proteins can be prepared by both chemical and physical methods. However major drawbacks are associated with the use of chemical crosslinking methods. Firstly, organic solvents used to make w/o emulsions may adversely affect the stability of proteins and, more importantly, the applied crosslinking agents can chemically modify proteins [84]. Secondly, complete removal of the unreacted and often toxic crosslinker is difficult to achieve. Recently, some chitosan nanoparticles were still crosslinked by
glutaradehyde [85, 86]. Unfortunately, the toxicity of glutaraldehyde on cell viability limits its utility in the field of drug delivery. So, the other method that is greatly interested to use for avoiding toxicity from the chemical cross-linking agent, is ionic cross-linking method. Ionic cross-linking is a popular technique due to its simplicity and mild condition. The technique involves the cross-linking between cationic molecules and anionic molecules via electrostatic interaction. The most commonly used cationic polymer is chitosan and the anionic material is tripolyphosphate (TPP).

![Figure 2](image)

**Figure 2** Preparation processes of chitosan microspheres by crosslinking method


### 2.3 Ionic cross-linking methods

A comparison with covalent crosslinking, ionic crosslinking has more advantages: mild preparation conditions and simple procedures. The complexation between chitosan-based polymers and oppositely charged macromolecules can be exploited to prepare micro/nanoparticles suitable for drug delivery. The particles are prepared by ionic crosslinking through self-assembly of chitosan/chitosan derivatives and oppositely charged macromolecules or by the addition of a low molecular weight anionic crosslinker, such as tripolyphosphate (TPP), sodium sulfate or cyclodextrin (CD) derivatives to chitosan solutions. The ionic crosslinking methods mentioned above have received much attention in recent years for the preparation of protein
formulations because they do not involve the use of chemical crosslinkers and avoid the use of organic solvents and high temperatures [26, 87-89]. As shown in Figure 3, ionic gelation of chitosan with TPP has been extensively used for the preparation of protein and antigen-loaded nanoparticles [88, 90-95]. Alonso et al. [88, 92] first reported TPP-crosslinked chitosan nanoparticles in 1997. TPP is non-toxic and has multivalent anions. It can form a gel by ionic interaction between positively charged amino groups of chitosan and negatively charged counterions of TPP [96]. In this process, an aqueous solution of chitosan is added drop-wise to an aqueous solution of TPP at ambient temperature under stirring as shown in Figure 4. Due to complexation of the oppositely charged components, chitosan nanoparticles are formed [91, 93]. Using this method, chitosan nanoparticles loaded with insulin and tetanus toxoid have been prepared and investigated as nasal delivery vehicles [91, 93]. Xu et al. [26] synthesized water-soluble chitosan derivative, N-(2-hydroxy) propyl-3-trimethyl ammonium chitosan chloride by the reaction between glycidyl-trimethyl-ammonium chloride and chitosan. Nanoparticles of 110–180 nm in size were formed based on ionic gelation process of the chitosan derivative and TPP. Bovine serum albumin, as a model protein drug, was incorporated into the nanoparticles with encapsulation efficiency up to 90%. In addition, Amidi et al. [90] prepared N-trimethyl chitosan nanoparticles by ionic crosslinking of N-trimethyl chitosan with TPP and evaluated their potential as a carrier system for the nasal delivery of proteins, ovalbumin. The nanoparticles had an average size of about 350 nm and a positive zeta potential. They showed a loading efficiency up to 95% and a loading capacity up to 50% (w/w). Fernandez-Urrusuno et al. prepared insulin-loaded chitosan nanoparticles with a size of 300–400 nm and a positive surface charge. The in vitro release of insulin was occurred in less than 3 h. However, this relatively fast release is not a disadvantage since the average residence time of nasally administered formulations never exceeds few hours [91]. In another study, tetanus toxoid (TT)-added chitosan nanoparticles, with an average size about 350 nm and a positive surface charge, showed a high loading efficiency (around 50–60%). In vitro release studies showed an initial burst followed by a sustained release of antigenically active toxoid for 16 days [93]. Recently, there have been many studies focusing on the mucosal delivery of proteins and vaccines using TMC nanoparticles prepared by ionic gelation [90, 94, 97-99]. Sun
and Wan synthesized O-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (O-HTCC), which carries two quaternary amines. Both chitosan and O-HTCC nanoparticles were prepared by ionic gelation with TPP. O-HTCC nanoparticles showed a decrease in size (from 500 to 100 nm) with increasing TPP concentration (from 1 to 4 mg/ml). This was in contrast to chitosan particles, which showed an increase in size from 10 to 50 nm with increasing TPP concentration, indicating a different colloidal behavior of these two nanoparticle formulations. This was explained by competition between three kinds of electrostatic forces that exists during gelation process: electrostatic attraction between the protonated amine of chitosan and/or –N+(CH3)3 of O-HTCC with the TPP anion and repulsion force between –N+(CH3)3 groups of the polymer. At low TPP concentration the repulsion forces exceeds the attraction forces yielding ‘loose’ nanoparticles with a relatively large size (500 nm). At high TPP, the attraction forces are stronger than the repulsion forces and relatively small and stable particles (100 nm) are formed. The opposite result is showed in high concentration of chitosan [18]. Importantly, the O-HTCC nanoparticles had substantially higher loading efficiency and capacity for BSA than the chitosan nanoparticles, which might be due to the higher charge density of O-HTCC, as compared to chitosan [100]. Amorphophallus konjac glucomannan and phosphorylated glucomannan–chitosan nanoparticles were prepared by ionic crosslinking with and without using (TPP) as a crosslinker. These nanoparticles exhibited high loading efficiencies for insulin and the immunomodulatory protein P1. Moreover, the release of the proteins could be modulated by the composition of the nanoparticles [101]. In other studies, negatively charged cyclodextran (CD) derivatives have been used as crosslinker or co-crosslinker to prepare chitosan nanoparticles [102, 103]. These negatively charged CD derivatives can also form complex and stabilize proteins and have the capacity to enhance nasal absorption of peptide drugs [102, 104]. In addition to ionic gelation method, coacervation/precipitation has been used to prepare a great variety of protein-loaded chitosan microparticles. In these methods, a coacervate, e.g. sodium sulfate, is added drop-wise to an acidic solution of chitosan under stirring and sonication to prepare ionically crosslinked particles. This method has been used to prepare chitosan-based microparticles loaded with interleukin-2 (IL-2) [105]. Although the
coacervation/precipitation method is more protein-friendly than the chemical crosslinking preparation technologies, sonication may harm the protein structure. Self-assembled polyelectrolyte complexes (PECs) have been recently investigated for protein delivery [87, 101, 106-110]. Oppositely charged polyelectrolytes can form stable intermolecular complexes [108]. These PEC nanoparticles are either positively or negatively charged, and they show a pH-dependent destabilization. Schatz et al. synthesized a partially N-sulfated chitosan. Upon acidification of an aqueous solution of this amphoteric chitosan, nanoparticles were formed by electrostatic interactions between the non-sulfated protonated amine groups of chitosan and the negatively charged N-sulfated chitosan amines. These polyelectrolyte complexes can be used for encapsulation of macro-molecules but loading and releases studies have not been reported [110]. Chitosan-based nanoparticles were prepared by electrostatic complexation of poly-γ-glutamic acid (γ-PGA) and chitosan. The particle size and zeta potential of the formed nanoparticles were mainly dependent on the volumes and concentration of the γ-PGA solution added to the chitosan solution. γ-PGA was selected as a negatively charged crosslinking agent because it has been shown that nanoparticles containing this polymer have the capacity to target hepatocytes [111]. In another approach, Mao et al. prepared polyelectrolyte complexes (PEC) formed from chitosan, (pegylated)-TMC and insulin. Complexation of the polymers and insulin occurred only above the pI (5.3) of insulin. PEC nanoparticles of insulin and chitosan were spherical, a smooth surface and their size was in the range of 200–500 nm. The characteristics of PEC nanoparticles were unaffected after lyophilization [87]. In other studies, chitosan–dextran sulfate PEC nanoparticles were prepared for delivery of insulin [106] or vascular endothelial growth factor (VEGF) [108]. Insulin-loaded PEC nanoparticles had an average size of ~500 nm, a negative surface charge and a loading efficiency of 85% [106]. VEGF nanocomplexes were prepared by ionic crosslinking with dextran sulfate [108]. The average size of the nanocomplex was ~250 nm and they had a negative surface charge. The nanoparticles showed a sustained release of the protein for 10 days. Boddohi et al. prepared PEC nanoparticles based on chitosan–heparin (chi-hep) and chitosan–hyaluronan (chi-ha) polycation–polyanion pairs [107]. The nanoparticles were spontaneously formed by addition of anionic polyelectrolytes to chitosan. The authors observed different
colloidal characteristics of negatively and positively charged PEC nanoparticles. For chi-hep particles, the width of size distribution and mean particles size increased significantly (from 350 to 660 nm) as the charge-mixing ratio approached one. The size increased because the net particle charge was minimal at this polymer ratio. At charge ratios far above one, chi-hep nanoparticles showed a smaller size (450 nm) which can be attributed to a higher particle charge and thus better colloidal stability. Positively charged chi-hep nanoparticles tended to aggregate with increasing amount of heparin. Schatz et al. reported that preparation of chitosan–dextran sulfate (DS) PEC nanoparticles was reduced in size after addition of more DS to positively charged chi-DS PEC nanoparticles [112]. Cui et al. [113] used carboxymethyl cellulose to complex chitosan to form stable cationic nanoparticles and investigated the topical application of these nanoparticles containing plasmid DNA as a potential approach to genetic immunization. Sarmento et al. [14] also prepared insulin-loaded nanoparticles by ionotropic pre-gelation of alginate with calcium chloride followed by complexation between alginate and chitosan. The same group probed the structural integrity of insulin after being entrapped into chitosan/alginate nanoparticles [114]. The results confirmed that no significant conformational changes of insulin occurred in terms of α-helix and β-sheet content. Du et al. [115] prepared carboxymethyl konjac glucomannan/chitosan nanoparticles under very mild conditions via polyelectrolyte complexation. Bovine serum albumin, as a model protein drug, was incorporated into the nanoparticles and the encapsulation efficiency and in vitro release behavior of the bovine serum albumin were investigated. The nanoparticles not only exhibited pH-responsive properties, but ionic strength-sensitive properties. Liu et al. [116] prepared heparin/chitosan nanoparticles by polyelectrolyte complexation. Entrapment studies of the nanoparticles were conducted using bovine serum albumin as a model protein. Zheng et al. [117] prepared chitosan/glycyrrhetic acid nanoparticles by polyelectrolyte complexation and studied the glycyrrhetic acid encapsulation efficiency and in vitro release. Stoilova et al. [118] prepared PEC nanoparticles between chitosan and poly(2-acryloylamido-2-methylpropanesulfonic acid) by mixing aqueous solutions of its components or by free radical polymerization on chitosan template. The nanoparticles (mean diameter 250 nm and monomodal distribution) were stable in acidic and neutral medium and dissociated at pH 8. Zheng
et al. [119] prepared anionic or cationic nanoparticles based on chitosan and polyaspartic acid sodium salt. Nanoparticle carriers made of bioadhesive natural biomaterials could prolong the residence time and therefore increase the absorbance of loaded drugs. In addition, there are abundant resources in nature, highly stable, safe, non-toxic, hydrophilic, biodegradable and low cost in their processing. Particularly, most of natural biomaterials have hydrophilic groups such as hydroxyl, carboxyl and amino groups, which could form non-covalent bonds with biological tissues (mainly epithelia and mucous membranes), forming bioadhesion [120]. For example, chitosan, starch, alginate and so on are good bioadhesive materials.

Figure 3  Ionic gelation of chitosan of chitosan and TPP
Figure 4  Schematic representation of preparation of chitosan particulate systems by Ionic cross-linking methods

3. Chitosan

Chitosan, a cationic natural biopolymer, produced from deacetylation of chitin [poly-β-(1-4)-N-acetyl-D-glucosamine] as shown in Figure 5 which can be extracted from crustaceans, insects, fungi, etc. (Table 3) [15-19, 121]. The degree of deacetylation of chitosan is from 40% to 98% and the molecular weight is ranges between 5,000 Da and 2,000,000 Da [40]. Chitin and chitosan possess reactive hydroxyl and amino groups, but chitosan is usually less crystalline than chitin then it is a semicrystalline polymer, and which makes chitosan more accessible to reagents [121, 122]. After heating, they decompose prior to melting, thus these polymers have no melting points [122]. The primary amine groups show special properties that make chitosan very useful for pharmaceutical applications. So, the advantages of using chitosan is biocompatible, biodegradable, low toxicity, good mucoadhesion and
Figure 5  Structures of chitin and chitosan  


Table 3  Source of chitin and chitosan

<table>
<thead>
<tr>
<th>Sea animals</th>
<th>Insects</th>
<th>Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annelida</td>
<td>Scorpions</td>
<td>Green algae</td>
</tr>
<tr>
<td>Mollusca</td>
<td>Spiders</td>
<td>Yeast (β-type)</td>
</tr>
<tr>
<td>Coelenterata</td>
<td>Brachiopods</td>
<td>Fungi (cell walls)</td>
</tr>
<tr>
<td>Crustaceans:</td>
<td>Ants</td>
<td>Mycelia Penicillium</td>
</tr>
<tr>
<td>Lobster</td>
<td>Cockroaches</td>
<td>Brown algae</td>
</tr>
<tr>
<td>Crab</td>
<td>Beetles</td>
<td>Spores</td>
</tr>
<tr>
<td>Shrimp</td>
<td></td>
<td>Chytridiaceae</td>
</tr>
<tr>
<td>Prawn</td>
<td></td>
<td>Ascomyces</td>
</tr>
<tr>
<td>Krill</td>
<td></td>
<td>Blastocladiaceae</td>
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</tbody>
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Progress in Polymer Science 31(7): 603-632.
membrane permeable enhancing properties by opening of tight epithelial cell junctions [20, 21, 123, 124]. Chitosan is soluble in an acidic solution such as formic, acetic, lactic, malic, citric, tartaric, glyoxylic, pyruvic, glycolic, malonic and ascorbic acids at pH below 6.4-6.5 [22, 23, 122]. The solution properties of chitosan is dependent not only on its average degree of deacetylation but also on the distribution of the acetyl groups along the main chain in addition to the molecular weight [121]. However, it is very few attained solubility in general organic solvents [125] and some binary solvent systems [126, 127]. Chemically modified chitin and chitosan structures resulting in improved solubility in general organic solvents have been reported by many workers [128-130].

3.1 Applications of chitosan

Chitosan has been used in many applications such as photography, cosmetic, artificial skin, food and nutrition, ophthalmology, water engineering, fat trapper, biotechnology and drug delivery system etc. as shown in Table 4 & Table 5 [124, 131, 132]. In addition, chitosan is greatly used as a biomaterial and as a pharmaceutical excipient in drug formulations [133-136]. Chitosan has been used for the preparation of mucoadhesive formulations [43, 137-139], for drug targeting systems [140], and for formulations that enhance the absorption of macromolecular therapeutics (peptides protein therapeutics and antigens as well as plasmid DNAs) [137-139, 141, 142]. These thiolated chitosans have shown in situ gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH [143-145]. The strong mucoadhesive properties of the thiolated chitosans make them particularly suitable carriers for prolonged protein delivery at the mucosal sites. The achievement of predictable and reproducible release of an agent into a specific environment over an extended period of time has much significant valuation. It creates a desired environment with optimal response, minimum side-effects and prolonged efficacy. Controlled-release dosage forms enhance the safety, efficacy and reliability of drug therapy. The release of drugs, absorbed or encapsulated by polymers, involves their slow and controllable diffusion from/ through polymeric materials. Production of slow release (SR) drugs by the pharmaceutical industry is now a matter of routine. Drugs covalently attached to biodegradable polymers or dispersed in a polymeric matrix of such macromolecules may be released by
erosion/degradation of the polymer. Therapeutic molecules, complexed by polymers, may also be released from gels by diffusion. Chitosan can be easily bioabsorbable with gel-forming ability at low pH [139]. Moreover, chitosan has antacid and antiulcer activities which prevent or weaken drug irritation in the stomach. Also, chitosan matrix formulations appear to float and gradually swell in an acid medium. All these interesting properties of chitosan make this natural polymer an ideal candidate for controlled drug release formulations [124, 136]. Moreover, chitin and chitosan were used as a diluents and a disintegration agent in tablet compression. Sawayanagi et al. reported the fluidity and compressibility of combined powders of lactose with chitin (lactose/chitin), with chitosan (lactose/chitosan) and potato starch with chitin (potato starch/chitin), and with chitosan (potato starch/chitosan). The disintegration properties of tablets made from these powders, in comparison with those of combined powders of lactose with MCC (lactose/MCC) and potato starch with MCC (potato starch/MCC) in order to develop new direct compression diluents, are also reported. Recently, chitosan has gained importance as a disintegration agent due to its strong ability to absorb water. It has been observed that chitosan contained in tablets at levels below 70% acts as a disintegration agent. Recently, Mi et al. [146] have reported alginate as an anionic polyelectrolyte to control the swelling and erosion rates of chitosan tablets in acidic media. For transdermal drug delivery system, Thacharodi and Rao [147-149] reported permeation-controlled transdermal drug delivery systems (TDS) using chitosan gel as the drug reservoir. Propranolol hydrochloride (prop-HCl) was used as a model drug and various chitosan membranes with different crosslink densities were used as drug release controlling membranes. The physicochemical properties of the membranes have been characterized and the permeability characteristics of these membranes to both lipophilic and hydrophilic drugs have been reported [147, 148]. Microcapsules/microspheres could be also prepared by using chitosan for controlled release of cimetidine in form of chitosan/gelatin network polymer microspheres. Generally, chitosan is known to have good complexing ability; the –NH2 groups on the chain are involved in specific interactions with metals which are concerned with complexation for the recovery of heavy metals from various waste waters [121]. In addition, the protonated amine groups giving positive charges can interact with the cell membrane resulting in a
structural reorganization of tight junction-associated proteins and followed by the opening of cell membrane. This leads to the permeation enhancing effect [135]. Many studies indicated that chitosan could be significantly increased transport of buserelin, 9-desglycinamide 8-L-arginine vasopressin and insulin in Caco-2 cell monolayers [150-152]. Artursson et al. reported that chitosan can increase the paracellular permeability of [14C] mannitol (a marker for paracellular routes) across Caco-2 intestinal epithelia [133]. Borchard et al. [153] investigated chitosan glutamate solutions at pH 5 and 7.4 for their effect to increase the paracellular permeability of [14C] mannitol and fluorescently labelled dextran (MW 4400 Da) in vitro in Caco-2 cells. No effect on the permeability of the monolayer could be observed, indicating that at neutral pH value of chitosan is not effective as permeation enhancer [153]. The pH dependency on chitosan’s effect on the epithelial permeability was further studied by Kotze et al. [154]. Two chitosan salts (hydrochloride and glutamate) were evaluated for their ability to enhance the transport of [C]mannitol across Caco-2 cell monolayers at two pH values, 6.2 and 7.4. At low pH both chitosans showed a pronounced effect on the permeability of the marker, leading to 25- (glutamate salt) and 36-fold (hydrochloride salt) enhancement. However, at pH7.4 both chitosans failed to increase the permeability, due to solubility problems [154]. In order to solve the problem of its solubility and using in other applications, modifications of chitosan structure are interesting in many researchers. The most position of chitosan structure has modified involving the –NH2 group at the C-2 position or non specific reactions of –OH groups at the C-3 and C-6 positions (especially esterification and etherification) as shown in Figure 6 [122]. The –NH2 in the C-2 position is the important point of difference between chitosan and cellulose. The main reaction easily performed involving the C-2 position is the quaternization of the amino group or a reaction in which an aldehydic function reacts with –NH2 by reductive amination. Carboxy-methyl-chitosan is the most fully explored derivative of chitosan; it is an amphoteric polymer, its solubility depends on pH [155] together with trimethyl-chitosan ammonium and chitosan-grafted copolymers have such the advantage of being water soluble [121, 156]. N-methylene phosphonic chitosans is an anionic derivative that has an amphoteric character for a good complexing efficiency for cations such as Ca2+, and those of transition metals (Cu (II), Cd (II), Zn (II) etc.)
In addition, cyclodextrin-linked chitosans are recently popular because of their ability to encapsulate hydrophobic molecules in their hydrophobic cavity [121]. However, a processing of modification of chitosan structure has a high cost, low final yield and using many steps for some derivatives when comparing with chitosan in salt form. Due to chitosan, as a polyelectrolyte, is able to form electrostatic complexes under acidic conditions with oppositely charged polymers: e.g. polyacrylic acid, sodium salt (PAA), carboxymethylcellulose (CMC) [159], xanthan, carrageenan, alginate (extracted from brown algae), pectin, heparin, hyaluronan (HA) [160, 161], sulfated cellulose, dextran sulfate, N-acylated chitosan/chondroitin sulfate [121] including proteins and DNA [121]. Many methods or results of electrostatic PEC between chitosan and synthetic or natural polymers are previously described above in section of ionic cross-linking method.

Table 4 Principal properties of chitosan in relation to its use in biomedical applications

<table>
<thead>
<tr>
<th>Potential Biomedical applications</th>
<th>Principal characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical sutures</td>
<td>Biocompatible</td>
</tr>
<tr>
<td>Dental implants</td>
<td>Biodegradable</td>
</tr>
<tr>
<td>Artificial skin</td>
<td>Renewable</td>
</tr>
<tr>
<td>Rebuilding of bone</td>
<td>Film forming</td>
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<tr>
<td>Corneal contact lenses</td>
<td>Hydrating agent</td>
</tr>
<tr>
<td>Time release drugs for</td>
<td>Nontoxic, biological</td>
</tr>
<tr>
<td>animals and humans</td>
<td>tolerance</td>
</tr>
<tr>
<td>Encapsulating material</td>
<td>Hydrolyzed by lyzosyme</td>
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<tr>
<td></td>
<td>Wound healing properties</td>
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<tr>
<td></td>
<td>Efficient against bacteria,</td>
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<tr>
<td></td>
<td>viruses, fungi</td>
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</table>

**Progress in Polymer Science** 31(7): 603-632.
Table 5  Principal applications for chitosan

<table>
<thead>
<tr>
<th>Application</th>
<th>Detailed Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agriculture</td>
<td>Defensive mechanism in plants</td>
</tr>
<tr>
<td></td>
<td>Stimulation of plant growth</td>
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<tr>
<td></td>
<td>Seed coating, Frost protection</td>
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<tr>
<td></td>
<td>Time release of fertilizers and nutrients into the soil</td>
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<tr>
<td>Water &amp; waste treatment</td>
<td>Flocculant to clarify water (drinking water, pools)</td>
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<tr>
<td></td>
<td>Removal of metal ions</td>
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<tr>
<td></td>
<td>Ecological polymer (eliminate synthetic polymers)</td>
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<tr>
<td></td>
<td>Reduce odors</td>
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<tr>
<td>Food &amp; beverages</td>
<td>Not digestible by human (dietary fiber)</td>
</tr>
<tr>
<td></td>
<td>Bind lipids (reduce cholesterol)</td>
</tr>
<tr>
<td></td>
<td>Preservative</td>
</tr>
<tr>
<td></td>
<td>Thickener and stabilizer for sauces</td>
</tr>
<tr>
<td></td>
<td>Protective, fungistatic, antibacterial coating for fruit</td>
</tr>
<tr>
<td>Cosmetics &amp; toiletries</td>
<td>Maintain skin moisture</td>
</tr>
<tr>
<td></td>
<td>Treat acne</td>
</tr>
<tr>
<td></td>
<td>Improve suppleness of hair</td>
</tr>
<tr>
<td></td>
<td>Reduce static electricity in hair</td>
</tr>
<tr>
<td></td>
<td>Tone skin</td>
</tr>
<tr>
<td></td>
<td>Oral care (toothpaste, chewing gum)</td>
</tr>
<tr>
<td>Biopharmaceutics</td>
<td>Immunologic, antitumoral</td>
</tr>
<tr>
<td></td>
<td>Hemostatic and anticoagulant</td>
</tr>
<tr>
<td></td>
<td>Healing, bacteriostatic</td>
</tr>
</tbody>
</table>

Progress in Polymer Science 31(7): 603-632.
4. Shellac

Shellac produced from lac insect *Laccifer Lacca* is abundant in Thailand, China and India. The resinous secretion can be purified to yield shellac which has been used in the food industry, paint industry and, in a lesser extent, in the pharmaceutical industry [28, 29]. Shellac is used as a moisture barrier for food and nutritional/health supplements. Further applications of shellac are enteric coating for food, retard coating for food and it also can be used as sub-coat or gloss coat. Shellac is particular interesting because it is one of the few excipients allowed for these coating purposes in food. In addition, it is natural material from renewable resources [162]. In pharmaceutical industry, shellac has excellent film forming and protective properties. Shellac begins to dissolve above pH 7.0 (the pKa of shellac is 6.9-7.5) which can provide an advantage to be used in the pH of the body fluid (pH 7.4) [30]. However, the low amount of carboxylic acid group on the shellac structure and the high pKa lead to the low solubility at pH below 7. The main structure of shellac is the combination of polyesters and single esters consisting of hydroxyl and carboxyl groups as shown in Figure 7 [28]. The details of shellac will be discussed as the following section.
4.1 lac insect

Lac is the insect which lives on trees called lac host trees where it secretes the lac resin or sticklac. Shellac is scrapped off and manufactured by lac. Lac insect considerably distribute in Tropical Asia such as India, Bangladesh, Myanmar, Thailand, South China, etc. Only 20 species of lac insect spread in the world. Nevertheless, only two species, Laccifer lacca in India and China and L. Chinensis in China and Thailand, have been used in many industries. One life cycle of a lac insect takes about half a year and consists of four stages: egg, larva, pupa and adult as shown in Figure 8. So, the lac industry could be produced only twice a year. In addition, lac insect can be very destructive to trees, stunting or killing twigs and branches of the
host trees by inserting their long suctorial mouthpart into the tree and draw out sap. Lac insects do not only drain out the sap but they also transmit germ. Therefore, we must realize lac insect is vermin for plant [163].

![Lac insect life cycle (Kerria lacca)](image)

**Figure 8** Lac insect life cycle (Kerria lacca)


### 4.2 Extraction of Lac

Lac cultivation is not necessary using any large investment requirement. Harvesting is done by removing the lac encrusted twigs. Stick lac is name of raw lac (Figure 9a). It composes of resin, insect body, sand, coloring matter (lac dye) and twig debris. The problem of stick lac is the decrease in quality and change into lump when keep in a long term of storage. However, this problem can be solved by keeping high moisture content of stick lac or converting into seed lac. Seed lac is a granular lac which prepared by grinding stick lac in a crude mortar, sieving to
remove sand and washing to wash out the lac dye and twig debris. The general appearances of seed lac are yellow or reddish brown and small seed (about 10 meshes or smaller) (Figure 9b). In addition, the seed lac is washed, melted, spread out in a thin layer film and dried. The product obtained is called “shellac” (Figure 9c) [164].

Figure 9 Stick lac (a), seed lac (b), shellac (c), and button lac (d)

4.3 Shellac processing

Handmade process, heat process or solvent process is the general method for produced shellac. Handmade process is a traditional process which produces shellac from seed lac. Seed lac is filled into long cloth bag and melts by charcoal-fired hearth to form molten lac. It is then squeezed from twisting the bag and
spread into thin sheets. Moreover, the molten lac is allowed to solidify in form of discs, and then it is known as “button Lac” (Figure 8d). Examples of handmade shellac are lemon one shellac, standard one shellac, superior shellac, etc. Second process is a heat process which is utilized in industrial level. The seed lac is liquefied by hot stream and the molten lac is filtered by hydraulic pressure machine. Shellac sheet is prepared by drying the filtered molten lac and then broken it in the form of pieces called “flakes”. Many grades of machine made shellac, for example, orange shellac, orange fine shellac, lemon one shellac, lemon two shellac, etc. Third process is a solvent process which concerns an organic solvent. Seed lac is dissolved in ethanol; impurity and wax are removed by filtering through filter. Alcohol is recovered and the residue shellac is stretched with a roller. The shellac products from this process are dewaxed platina, dewaxed blonde, dewax lemon, or dewax orange shellac. Moreover, bleached shellac is produced by solvent process with base solution. The bleached shellac is prepared by dissolving seed lac in sodium carbonate solution at a high temperature, stirring with bleaching agent (such as sodium hypochlorite) and then filtering after cooling. The commercial bleached shellac is dewaxed bleached shellac and waxy bleached shellac.

Following to the United States Pharmacopoeia-National Formulary (USP 30-NF 25), shellac is categorized into four grades: orange, dewaxed orange, regular bleached and refined wax-free bleached shellac (United States Pharmacopeial Convention 2007: 3417). The difference in grades depending on the processing of seed lac is treated. The evaporation of filtered ethanolic solutions of seed lac is the processing for obtained orange shellac and it could become dewaxed orange shellac through a filtration process. Regular bleached shellac is obtained by the method described above. The resin is removed by sulfuric acid precipitation after bleaching process. Refined wax-free bleached shellac is required another filtration step to remove the waxes.

4.4 Chemical structure of shellac

The exact molecular structure of shellac is unknown. A network of hydroxy fatty acid esters and sesquiterpene acid esters were appeared on the composition of shellac. Shellac usually consists of odorous compound, wax and a mixture of dyes such as erythrolaccin and desoxyerythrolaccin
(hydroxyanthraquinone derivatives). Three main compositions of shellac are hard resin, soft resin and wax, the resins consists of hydroxyl groups. The composition of shellac depended on the source and harvest time of the stick lac resulting in the variation in physical property which is a problem for commercial use. For example, the melting point is reported in the range of 77°C to 120°C [165], specific gravity is between 1.14 to 1.21, AV is 65-75 and saponification value is 220-230 [166].

After alkaline treatment [166], shellac displays two main compounds that are aliphatic and terpenic acids. The proportion between aliphatic and terpenic acids is about 50:50. The aliphatic acids are insoluble in water; the main constituent is aleuritic acid (35%) while the terpenic acids are easily soluble; the main constituent is jalaric acid (25%). Other acids are shellolic/epishellolic, laccijalaric and butolic acid.

4.5 Properties and applications of shellac
4.5.1 Low water vapor permeability

Moisture-protective polymer should be prepared film which can prevent water vapor from entering the coated products. Several studies reported that shellac had the better effective barrier to water vapor, when compared to other polymers [167, 168]. Therefore this characteristic of shellac is the important reason for utilizing as an excellent moisture barrier.

4.5.2 pH-dependent solubility

Because the structure of shellac consists of carboxyl groups, shellac is not dissolved at the acidic pH of the stomach and dissolved at a higher pH of the intestine (above pH 7.0) [169]. Therefore, shellac could be applied as enteric coating polymer. In addition, it can be soluble in alcohol and alkaline solution such as ammonia, sodium borate, sodium carbonate, and sodium hydroxide, and also in other organic solvents.

4.5.3 Gloss

Gloss is an optical property, which is based on the interaction of light with physical characteristics of a surface. The factors that affect gloss are the refractive index, the surface topography and the angle of incident light. Shellac has high refractive index (1.5210-1.5272) and gloss [170], so shellac is extensively applied for coating of woodwork and fruit because of this attractive property.
4.5.4 Low thermal conductivity

Shellac has a low thermal conductivity. It also has excellent dielectric properties, high dielectric strength, a low dielectric constant and a good tracking resistance [171]. Shellac has been used as an insulator for several decades.

4.5.5 Good adhesive property

Alkaline solution and alcoholic solution of shellac provide spreadable films of high adhesive power. They display a good adhesion to a wide range of surface except teflon and silicone coated glass [172]. Based on these properties, shellac has been developed for various applications. Shellac is often used as a finish for fine furniture, an aqueous varnish for paper, wood and leather and an additive for some cosmetic products. In pharmaceutical applications, shellac has been used for a long time for protective coating, enteric coating, microencapsulation and matrix forming agent for tablet [165]. Since 1930, shellac has been used for enteric coating. Shellac was dissolved in ammonia solution providing a good enteric coating as following by report of Wruble in 1930. However, the drawbacks of shellac as a coating material are batch to batch variationthe low solubility and aging effect upon storage. Aging effect is a resulting from polymerization of transesterification of the hydroxyl group of shellac molecule with the carboxyl group of other adjacent shellac molecule leading to the delayed disintegration and reduced solubility of shellac [173]. The low amount of carboxylic acid group per shellac molecule and the high pKa lead to the low intestinal solubility which is the other disadvantage of shellac. Since the pH in the small intestine is in the range of 3.8-6.9, then shellac-coated tablets cannot be to disintegrate at that pH, which most drug release is required [174]. This is the reason of declining into disfavor as the enteric coating material [165]. Pearnchob et al. developed the shellac-coated soft gelatin capsules that demonstrated ion faster disintegration in phosphate buffer pH 6.8 by the addition of pore-formers, such as organic acids and hydrophilic polymer. Ethanolic and aqueous shellac solutions were prepared and comparatively studied. Sorbic acid as a pore-former showed the best disintegration in this experiment. The disintegration time of ethanolic shellac-coated soft gelatin capsules decreased with increasing amount of pore-former. The addition of all pore formers showed that the disintegration times in phosphate buffer pH 6.8 significantly decreased but the behavior of shellac-coated soft gelatin capsules in 0.1
N HCl remained unchanged. The addition of hydroxypropyl methylcellulose (HPMC) capsules could be also decreased the longer disintegration time of aqueous shellac-coated soft gelatin [167]. Qussi and Suess [175] also investigated aqueous shellac-coated pellets containing different amounts of polyvinyl alcohol, hydroxypropyl methylcellulose, and carbomer. The results indicated that all material coating systems could protect the dissolution of drug in simulated gastric juice for 2 h and rapid release of drug within 45 min in simulated intestinal fluid. Moreover, polyvinyl alcohol and hydroxypropyl methylcellulose exhibited a positive effect on the stability of shellac-coating systems after storing at room temperature and 40-45 % RH for 3-12 months [175]. Limmatvapirat et al. attempted to improve the mechanical properties and solubility of shellac by partial hydrolysis with 2% w/w sodium hydroxide solution at various times. They found that the higher prolongation of hydrolysis contributed to the higher acid value (AV), resulting in the higher solubility of shellac in buffer solution. The AV was influenced by the amount of free carboxylic group and the ionization constant (pKa). However the pKa of hydrolyzed shellac did not change (6.5-6.8), so the increment of AV should be affected by an increase in free carboxyl groups. As compared to the other enteric polymers, shellac showed the lower aqueous solubility due to the higher pKa [30, 176]. The results demonstrated that the solubility of hydrolyzed shellac increased with increasing hydrolysis time. The improved solubility was well corresponded with the increase in AV, suggesting the solubility increment by ionization of more carboxylic groups. Although the partially hydrolyzed shellac showed better solubility and flexibility properties compared with native [30], the stability problem was not yet solved. The enhancement of flexibility was related with the increase in soft resin of shellac [30].

The instability of shellac is the main problem due to the polymerization between hydroxyl and carboxyl groups on the molecular structure when it keeps for a long time [177-179]. There have been several attempts to solve the stability problem. Plasticizer is one of the methods to improve the stability of polymer. Plasticizers can reduce the intermolecular forces and increase in the mobility of the biopolymer chains then improving the mechanical properties such as reducing the rigidity of the film structure. [180-183]. Luangtana-anan et al. investigated the types of plasticizers on stability of shellac film. Diethyl phthalate (DEP), triacetin (TA) and polyethylene
glycol 400 (PEG 400) were used in this study at 10% w/w concentration. The addition of PEG 400 gave the highest stability indicating by the amount of insoluble solid. The high stability of shellac plasticized with PEG 400 might be a result of the interference of a larger molecule of PEG 400 causing the difficulty in interaction among the active groups of shellac enabling to the less polymerization. In addition, the result might be due to the retaining of PEG400 within shellac network as compared with other plasticizers because of the higher molecular weight and less volatilization. [179]. The effect of molecular weight of molecular weights of PEG (PEG 200, 400 and 4000) had an influence on the stability of shellac films [184]. Proposed diagram of unplasticized and plasticized shellac film with PEG as showed in Figure 10. The protection of PEG200 and 400 were the result of the hydrogen bonding between hydroxyl groups of PEG and carboxyl or hydroxyl group of shellac. However, the PEG 4000 could not protect shellac film from the polymerization due to the longer chain of PEG 4000, causing the difficulty in insertion between the polymer networks. The result indicating that the different molecular weights of plasticizer had an important role on the stability of shellac. Apart from the molecular weight of plasticizer, the concentration of plasticizer also had an effect on polymerization. Therefore, the application of optimum molecular weight and concentration of plasticizer were a requirement for the protection of shellac from the polymerization [184]. In addition, the compositied solvent was used to dissolve shellac with purpose to increase solubility and stability of it. The shellac samples were prepared by dissolving with 1-amino-2-methyl-1-propranol (AMP) and ammonium hydroxide (AMN) for different ratio of AMP: AMN. The results demonstrated that shellac was partially dissolved at pH 7.0 and completely dissolved at pH 7.3 and higher. The percent dissolved of shellac salts was significantly increased while the dissolving time was significantly decreased with increasing AMP content. AMP might interact more strongly with the carboxylate, resulting in higher ionization, plasticization and stability compared with AMN. The increase in the interaction was attributed to the hydrogen bond formation between hydroxyl groups of AMP and shellac. In addition, the steric effect of the large molecular size of AMP might cause the polymer chains to
Figure 10  Proposed diagram of unplasticized and plasticized shellac film with PEG (200, 400 4000) after storage at 40 °C, 75% for 3 months

separate from each other and reduce the possibility of esterification among polymer chains of shellac. The proposed diagram of salt formation of various forms of shellac as showed in Figure 11. In addition, shellac was modified with cyclic anhydride such as succinic acid to improve the solubility and stability of shellac by increasing the number of carboxyl groups. Limmatvapirat et al. reported that the formation of shellac succinate was obtained by organic solvent reaction. Various techniques were used to characterize the interaction between succinic acid and shellac structure. The increase in succinate amount to shellac polymer affected the film properties such as
mechanical properties, pH solubility, water vapor permeability and stability. In addition, the enhancement of aqueous solubility at the pH of small intestine is observed, indicating that the modification of shellac could enhance the stability and solubility of shellac for enteric coating application [29].

Figure 11  Proposed diagram of salt formation of various forms of shellac  

5. Drug uptake & transport  
Cell monolayers can be used to identify drugs with potential absorption problems and possibly applied to predict drug absorption in vivo [185]. Since drug transport studies in cell monolayers are easy to perform and require only small quantities of drugs, they have been suggested for the screening
of drug absorption at an early stage in the drug development process. The transport of drugs across the intestinal epithelium may occur by one or more of four different routes: the passive transcellular and paracellular routes, the carrier mediated route and by transcytosis (Figure 12). Caco-2 monolayers have been used to study drug transport of all four routes.

Since the surface area of the brush border membranes is > 1000-fold larger than the paracellular surface area [186], it can be assumed that these drugs are transported exclusively by the passive transcellular route. Drugs that are slowly and incompletely passively absorbed such as hydrophilic drugs and peptides, distribute poorly into cell membranes. It is therefore generally assumed that these drugs are transported through the water-filled pores of the paracellular pathway across the intestinal epithelium. It is possible that even very hydrophilic drugs may be transported partly by the transcellular route [187]. However, transcytosis of macromolecules and even small microparticles is more effective in M-cells, specialized epithelial cells overlying the lymphoid tissue of the intestinal epithelium [188]. This cell type, which may have lower proteolytic activity in its transport vesicles, is the main target for antigen-containing microparticulate delivery systems intended for oral vaccination. The Caco-2 cell is a widely used in vitro model for small intestinal absorption because they spontaneously differentiate into monolayers of polarized cells that resemble intestinal enterocytes when cultured on permeable inserts [189]. The properties of Caco-2 cells in one laboratory may therefore differ from those in another. The properties of Caco-2 monolayers also vary with time within a laboratory, e.g. with the passage number [190], the time in culture [191] the extracellular (filter) support [192] and the cell culture medium [193]. In summary, the results obtained to date indicate that Caco-2 monolayers can be used to predict drug transport by different pathways across the intestinal epithelium but that the best correlation with the absorbed fraction in vivo is obtained for passively transported drugs. Therefore, Caco-2 monolayers can be used as a simple reference model in predictions of passive drug absorption.

In addition, Caco-2 cells have widely been used to evaluate the cytotoxicity for several drugs or compounds. The one popular technique for assessing the cytotoxicity of compounds is MTT test which determines the effects of the
compounds on intracellular dehydrogenase activity. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a water soluble yellow tetrazolium salt that is changed to insoluble purple formazan by active mitochondrial succinate dehydrogenases in living cells.

Figure 12 Schematic of an intestinal epithelium; The arrows indicate the four different drug transport routes: 1) the passive transcellular, 2) the passive paracellular, 3) the active carrier-mediated transcellular, and 4) the transcytosis routes


Ma & Lim evaluated the response of Caco-2 cells to chitosan molecules and chitosan nanoparticles and to relate this to the capacity of the chitosan formulations to mediate insulin transport across cell monolayers [189]. The effect of incubation time, temperature and loading concentration of nanoparticles were investigated in this study. Rapid uptake was observed in the first 30 min of contact, followed by a period of inactivity in the next 30 min, and then a period of increased
cellular uptake in the subsequent 1 h. Uptake of nanoparticle was concentration dependent, the 2-h uptake increasing by 2.07-fold, from 35.99 to 74.36 g/mg, when the loading concentration was increased from 0.33 to 1.00 mg/ml. Temperature had a significant effect on the cellular uptake of the nanoparticle. The 2-h uptake of the nanoparticles at the loading concentration of 1 mg/ml was reduced by 7.6-fold, from 89.9 ± 17.5 to 11.8 ± 2 g/mg, when the uptake temperature was lowered from 37 °C to 4°C. Temperature affected the nanoparticle uptake at all the loading concentrations studied. Hence, uptake of nanoparticle by the Caco-2 cell monolayers was dependent on time, temperature and loading concentration. Sandri et al. prepared nanoparticle from trimethyl-chitosan and with TPP and loaded with fluorescein isothiocyanate dextran (FD4, MW4400 Da), used as the model macromolecule. The permeability studies by Caco-2 cell monolayer indicating that nanoparticulate system was significantly higher than that of control (p < 0.01) (control=FD4 solution) [98]. Sadeghi et al. studied the effect of various types of quaternized derivatives of chitosan: trimethyl chitosan (TMC), dimethylethyl chitosan (DMEC), diethylmethyl chitosan (DEMC) and triethyl chitosan (TEC) on the permeability of insulin across intestinal Caco-2 monolayers and compared with chitosan both in free-soluble form and in nanoparticulate systems. Transport studies of insulin together with the soluble polymers across Caco-2 cell layers showed the following ranking: TMC > DMEC > DEMC > TEC > chitosan which is in agreement with the strength of the cationic charge of the polymer and a similar rank order was also observed for the zeta potentials of the various polymers in solution form. However, the transport efficiency of insulin in free form, in the presence of free-soluble polymers, across the Caco-2 cell monolayer, was shown to be higher than its loaded form in nanoparticles prepared with the same chitosan derivatives. Their study clearly showed that the chitosan derivatives in free-soluble form had higher positive surface charge and could be consequently considered as good permeation enhancers for hydrophilic drugs through the paracellular route in suitable drug delivery systems. The chitosan derivatives in nanoparticle form have less positive surface charge and their interactions with tight junction are limited and hence the drug transport across the monolayer is more likely through the transcellular pathway rather than by tight junction opening [194]. Liu et al. developed the lipid nanoparticle for the oral delivery of bufalin (a hydrophobic
active component extracted from the traditional Chinese medicine Chan’su) by grafted with wheat germ agglutinin (WGA). The high-pressure homogenization was used for preparing the lipid nanoparticle. The association of WGA-grafted lipid nanoparticles was approximately 1.5-fold higher than that of WGA-free nanoparticles or BSA-grafted nanoparticles. This indicated that WGA facilitated the association between lipid nanoparticles and cells [195]. Beck et al. prepared nanoparticle-coated microparticles by spray-drying using polymeric colloidal suspensions as coating material. Transport studies across Caco-2 cell monolayers also demonstrated the feasibility of these microparticles to control the absorption of dexamethasone across the Caco-2 cell lines depending on the type of the coating material. Nanoparticle-coated microparticles presented lower permeability values compared to the free dexamethasone solution. In accordance with the in vitro drug release data, the nanoparticle-coated microparticles presented the more pronounced delay in the absorption of dexamethasone across Caco-2 cells. Therefore, the rate limiting barrier to drug absorption is not only the factor of epithelial permeability but also the drug release played an influence depending on the type of formulations. Hence, in vitro studies on Caco-2 monolayers appear as a useful tool in the development of microparticulate/nanoparticulate oral controlled release forms prior to their evaluation in clinical studies [196].

6. Stability

In last decade, nanoparticles have widely been investigated for delivering several drugs or proteins to improve oral bioavailability, to sustain drug effect in target tissue, to solubilize drugs for intravascular delivery, and to improve the stability of therapeutic agents against enzymatic degradation. However, the major obstacle that limits the use of these nanoparticles is due to the physical instability (aggregation/particle fusion) and/or to the chemical instability (hydrolysis of polymer materials forming the nanoparticles, drug leakage of nanoparticles and chemical reactivity of medicine during the storage). These instabilities are frequently observed when the nanoparticles are stored for an extended periods [197].

Luangtana-anan et al [198] investigated the stability of protein-loaded chitosan microparticles under storage at 25°C for 28 days. A comparison was made
between those preparations with PEG 200 and without PEG 200. It was found that the stability decreased upon storage and the aggregation of microparticles could be observed for both preparations. The reduction in the zeta potential and the increase in the pH, size, and loading capacity were observed when they were kept at a longer period. The significant change of those preparations without PEG 200 was evident after 7 days of storage whereas those with PEG 200 underwent smaller changes with enhanced stability after 28 days of storage. The stabilization of PEG 200 could be due to the steric hindrance effect of PEG 200. In addition, it was found that the larger size was related to the change in pH of the chitosan solution. The stability of the microparticles could be due to the electrostatic charge of particles. The repulsion forces of the particles were attributed to the stability of small particles. The reduction of electrostatic charge until a certain extent could lead to the aggregation [20]. The result was similar to those in a number of reports which found that the stabilization of the particles was due to the electrostatic nature of the chitosan particles [20, 106]. Sarmento et al. [106] found that insulin-loaded chitosan nanoparticles changed in size and zeta potential due to the aggregation after 28 days of storage. So, two main factors that had an influence on the instabilities of nanoparticles for a long-term storage are physical and chemical factors such as pH, type of polymer and temperature.

6.1. Physical factors

The thermal motion of the particles in the colloidal size range is known as Brownian motion. As a result of thermal motion, colloidal particles diffuse from a region of high concentration to a region of lower concentration until the concentration is uniform throughout. Gravitational forces, which cause particles to sediment, and Brownian motion (diffusion forces), oppose one another. Both forces are related to the particle size.

Colloids are the size range at which the Brownian forces dominate the gravitational forces, so they tend to remain suspended [199].

6.2. Chemical factors

The chemical stability of colloidal polymeric carrier is dependents on their storage conditions (the temperature and the pH medium) and on the exact
composition of the formulation stored (the type and the molecular weight of the polymer used in preparing nanoparticles).

6.2.1. Effect of polymer type

It is well known that nanoparticles made of hydrolytic degradable polymers will be degraded (although at a low rate if the temperature and pH are controlled) over time. Lemoine et al. [200] have found that the stability of polymeric nanoparticles depended on the type of polymer with the following increasing order of polymer stability: poly(D,L-lactide-co-glyco-lide) PLA25GA50 (50% D,L-lactide acid and 50% Glycolide acid) < poly(D,L-lactide-co-glycolide) PLA37.5GA25 (75% D,L-lactide acid and 25% Glycolide acid) < poly(D,L-lactide) PLA50=poly(ε-caprolactone) PCL. Furthermore, for PCL nanoparticles, the initial molecular weight of the polymer did not influence the degradation profile.

6.2.2. Effect of pH of the aqueous dispersion

The in vitro degradation of nanospheres made from poly(D,L-lactide) of two different molecular weights (Mw: 25000 and 95000) has been investigated [201]. It has been found that the aqueous dispersion pH of nanospheres has major effects on the chemical stability of the polymer. The best stability in aqueous medium was observed in a buffered solution with a pH corresponding to the physiological conditions and a temperature of 4 °C.

6.2.3. Effect of storage temperature

The storage temperature has a crucial effect on the long-term stability of nanoparticles. For example, when poly(D,L-lactide) and poly(ε-caprolactone) nanoparticles were stored for 350 days at 5 °C, only minor changes in the molecular weight of the polymers and in nano-spheres size were observed [202]. At 37 °C, there was a rapid degradation of both polymers in the dispersion. However, when PLA nanoparticles were stored at 25 °C, changes in the polymer molecular weight were detected after 4 month storage period. Similar results have been obtained by Lemoine et al. [200]. These authors have found that PCL and PLA50 nanoparticles can be kept at 4 °C and room temperature during one year.

In order to improve the physical and chemical stability of these systems, water has to be removed. Freeze-drying is the most commonly used process
which allows converting solutions or colloids into solids of sufficient stability for
distribution and storage in the pharmaceutical field for a long time.

6.3 Freeze-drying

Freeze-drying, also known as lyophilization, is an industrial process
which consists of removing water from a frozen sample by sublimation and
desorption under vacuum. However, this process generates various stresses during
freezing and drying steps. So, protectants are usually added to the formulation to
protect the nanoparticles from freezing and desiccation stresses. The objectives for
freeze-drying process of nanoparticles are the following: I) an elegant lyophilizate,
rapid reconstitution time of the suspension, II) a conservation of the physicochemical
characteristics of the freeze-dried product (a small or unmodified nanoparticle size,
and the drug entrapment), III) a less residual humidity <2%), IV) and also a good
long-term stability of the formulation [199].

6.3.1 Freeze-drying process

Freeze-drying is a widely used process for drying and
improving the stability of various pharmaceutical products including: viruses,
vaccines, proteins, peptides, or colloidal carriers: liposomes, nanoparticles,
nanoemulsions. This process is relatively slow and expensive, it is especially applied
only for products having a high added value. Freeze-drying cycle can be divided into
three steps: freezing (solidification), primary drying (ice sublimation) and secondary
drying (desorption of unfrozen water). Freezing step involves the liquid suspension
becomes more concentrated, its viscosity increases inducing inhibition of further
crystallization. The small percentage of water that remains in the liquid state and does
not freeze is called bound water. The primary drying stage involves sublimation of ice
from the frozen product and secondary drying involves the removal of absorbed water
from the product.

6.3.2 Freeze-drying of nanoparticles

A freeze-dried nanoparticle should have characteristics
requirement including: I) the preservation of the primary physical and chemical
characteristics of the product, II) an acceptable relative humidity, and III) long-term
stability. To meet these requirements, the component of the formulation is importance
as described below:
6.3.2.1 Importance of the formulation

Many components of the nanoparticles formulation have a crucial effect on the resistance of nanoparticles to the different stresses during freeze-drying. They are the type and the concentration of cryoprotectant, the nature of surfactant, the chemical groups attached to the nanoparticles surface, or the polymer used to form the nanoparticles.

6.3.2.2 Use of cryo and lyoprotectant

Freeze-drying may generate many stresses that could destabilize colloidal suspension of nanoparticles, especially, the stress of freezing and dehydration. It is well known that during the freezing of a sample there is a phase separation into ice and cryo-concentrated solution. In the case of nanoparticles suspension, the cryo concentrated phase is composed of nanoparticles and the other components of the formulation such as free surfactants, buffers, and unloaded drugs [203]. This high concentration of particulate system may induce aggregation and in some cases irreversible fusion of nanoparticles. Furthermore, the crystallization of ice may operate a mechanical stress on nanoparticles leading to their destabilization. For these reasons, special excipients must be added to the suspension of nanoparticles before freezing to protect these fragile systems [204]. These excipients are usually added in order to protect the product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) and also to increase its stability upon storage. Table 6 presents some examples of the excipients commonly used in freeze-drying process of pharmaceutical products with the presentation of their different role. The most popular cryoprotectants encountered in the literature for freeze-drying nanoparticles are sugars: trehalose, sucrose, glucose and mannitol (Table 7). These sugars are known to vitrify at a specific temperature denoted Tg′ [199]. The immobilization of nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. Generally, freezing must be carried out below Tg′ of a frozen amorphous sample or below Teu (eutectic crystallization temperature) which is the crystallization temperature of soluble component as a mixture with ice, if it is in a crystalline state in order to ensure the total solidification of the sample [205].
Table 6  Examples of commonly used excipients in freeze-drying of pharmaceutical products

<table>
<thead>
<tr>
<th>Type</th>
<th>Function</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulking agents</td>
<td>Provide bulk to the formulation specially when the concentration of product to freeze dry is very low.</td>
<td>Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine.</td>
</tr>
<tr>
<td>Buffers</td>
<td>Adjust pH changes during freezing.</td>
<td>Phosphate, tris HCl, citrate, and histidine.</td>
</tr>
<tr>
<td>Stabilizers</td>
<td>Protect the product during freeze-drying against the freezing and the drying stresses.</td>
<td>Sucrose, lactose, glucose, trehalose, glycine, sorbitol, mannitol, and PVP.</td>
</tr>
<tr>
<td>Tonicity adjusters</td>
<td>Yield an isotonic solution and control osmotic pressure.</td>
<td>Mannitol, sucrose, glycine, glycerol, and sodium chloride.</td>
</tr>
<tr>
<td>Collapse temperature modifiers</td>
<td>Increase collapse temperature of the product to get higher drying temperatures.</td>
<td>Dextran, hydroxypropyl-β-cyclodextrin, PEG, poly(vinyl pyrrolidone).</td>
</tr>
</tbody>
</table>


Trehalose seems to be a preferable cryoprotectant for biomolecules. It has many advantages in comparison with the other sugars such as less hygroscopicity, an absence of internal hydrogen bounds allowing more flexible formation of hydrogen bonds with nanoparticles during freeze-drying, very low chemical reactivity and finally, higher glass transition temperature $T_g'$ [206].

In general, the type of cryoprotectant must be selected and its concentration must be optimized to ensure a maximum stabilization of nanoparticles. Usually, a freeze-thawing study should be realized before freeze-drying to select the best cryoprotectant which is able to conserve the properties of nanoparticles. Another explanation of the mechanism of nanoparticles stabilization by cryoprotectants during the freezing step is the particle isolation hypothesis. It has been proposed that sugars isolate individual particles in the unfrozen fraction, thereby preventing aggregation during freezing above $T_g'$ [207]. A suggested stabilization mechanism of nanoparticles by lyoprotectants during drying steps is the water replacement hypothesis which was already explained the stabilization of liposomes and proteins [208, 209]. This mechanism supposes the formation of hydrogen bonds between a lyoprotectant and the polar groups at the surface of nanoparticles at the end of the drying process. These lyoprotectants preserve the native structures of
nanoparticles by serving as water substitutes. The amorphous state of nanoparticles and a lyoprotectant allows maximal H-bonding between nanoparticles and stabilizer molecules. So, the crystallization of this stabilizer can limit the formation of hydrogen bonds [203].

Table 7  Some of cryoprotectants used for the freeze-drying of nanoparticles

<table>
<thead>
<tr>
<th>Cryoprotectant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Trehalose</td>
</tr>
<tr>
<td>Lactose</td>
</tr>
<tr>
<td>Mannitol</td>
</tr>
<tr>
<td>Sorbitol</td>
</tr>
<tr>
<td>Aerosil (colloidal silicon dioxide)</td>
</tr>
<tr>
<td>Maltose</td>
</tr>
<tr>
<td>Poly(vinyl pyrrolidone)</td>
</tr>
<tr>
<td>Fructose</td>
</tr>
<tr>
<td>Dextran</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>Gelatine</td>
</tr>
</tbody>
</table>


6.4 Physico-chemical characterization of freeze-dried product

It is very important to characterize the freeze-dried matrix and to investigate the conservation of the nanoparticle properties. Furthermore such characterization may validate the applied conditions of the process and the optimized formulation. The most useful methods of characterization of freeze-dried matrix and nanoparticles are macroscopic aspect of freeze-dried product, nanoparticles size, zeta potential, and determination of drug content. The measurement of zeta potential is a good method to evaluate the state of nanoparticles surface and to detect any eventual
modification after freeze-drying [198]. Furthermore, it can be used to study the interaction between the cryoprotectant molecules and the nanoparticles surface.
CHAPTER 3
MATERIALS AND METHODS

1. Materials
   1. Ammonia solution 25% (Lot No. K39606032910, Merck, USA)
   2. Chitosan 35 kDa, 85%DD (Seafresh Co. Ltd., Thailand)
   3. Chitosan 45 kDa, 85%DD (Seafresh Co. Ltd., Thailand)
   4. Chitosan 200 kDa, 85%DD (Seafresh Co. Ltd., Thailand)
   5. Ethyl alcohol absolute (Lot No. J32T04, Mallinckrodt, Malaysia)
   6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Lot No. DU21373, Bio Basic Inc., Canada)
   7. Shellac (Thananchai Part., Ltd., Thailand)
   8. Sodium chloride (Lot No. 0811292, Ajax Pty Ltd, New Zealand)
   9. Sodium hydroxide (Lot No. B0274298, Merck, USA)
   10. Ammonium persulfate (Lot No. 1610700RevF, BIOLAB, USA)
   11. Coomassie Brilliantblue G250 (Lot No. k17788382, Merck, Germany)
   12. Glycine, USP Grade (Lot No. Z77377, RESEARCH ORGANICS, USA)
   13. TRIS biotechnology Grade (Lot No. 1846B027, RESEARCH ORGANICS, USA)
   14. Acrylamide (Lot No. 42106711 12501, Fluka, Switzerland)
   15. N, N'- Methylene-bisacrylamide (Lot No. 39815111 22100, Fluka, Switzerland)
   16. Potassium sodium (+) – tartrate (Lot No. AF504110, Ajax Pty Ltd, Australia)
   17. Sodium carbonate anhydrous (Lot No. AF405220, Ajax Pty Ltd, Australia)
   18. Copper (II) sulphate (Lot No. AF502341, Ajax Pty Ltd, Australia)
19. L-Glutamic acid (Lot No. AF609071, Ajax Pty Ltd, Australia)
20. Potassium dihydrogen phosphate (Lot No. 5H050017F, CARLO ERBA, France)
21. D-(-)-Glucose (Lot No. 0001420414, Sigma, USA)
22. Potassium bromide (Lot No. B0351607 905, Merck, Germany)
23. Formamide (Lot No. 0808223, Ajax Pty Ltd, Australia)
24. Ethylene glycol (Lot No. 1341646 23707040,Fulka, Belgium)
25. D-(-)-mannitol (Lot No. K909489888, Merck, Germany)
26. D-(-)-Trehalose dehydrate (Lot No. 0001434144, Fluka, United Kingdom)
27. Distilled water (Lot No. 0908061, General Hospital Product Public Co., Ltd., Thailand)
28. Bovine serum albumin (Lot No. S31292048, Fluka, Switzerland)
29. Hank’s Balanced Salt Solution (HBSS, Lot No. RNBB2942, Invitrogen, USA)
30. Phosphate buffered saline (Tablet, Lot No. 059k8215, Sigma, USA)
31. Trypan blue stain 0.4% (Lot No. J120-6, JR Scientific Inc., USA)
32. Dimethyl sulfoxide (DMSO, Lot No. RNBB8470,Sigma, USA)
33. Minimum Essential Medium Eagle (EMEM, Lot No. RNBB6109, Invitrogen, USA)
34. Sodium pyruvate (Lot No. RNBB9457, Invitrogen, UK)
35. Non-essential amino acid solution (Lot No. RNBB 8565, Invitrogen, USA)
36. Fetal bovine serum (Lot No. 8105552, Invitrogen, Australia)
37. Penicillin streptomycin (Lot No. 031M0787, Invitrogen, USA)
38. L-Glutamine (Lot No. RNBB0222, Invitrogen, UK)
39. 0.25 % Trypsin-EDTA solution (Lot No. 11D359, Invitrogen, UK)
40. Thiazolyl blue tetrazolium bromide (Lot No. 08797HJ, Sigma, USA)
41. Triton X-100 (Lot No. 057K00161, Sigma, Germany)
2. Equipments

1. Aluminium covers (Perkins, P/N SSC0000E030 open sample pan, Japan)
2. Aluminium crimping (Perkins, P/N SSC0000E032 crimping cover, Japan)
3. Analytical balance (Sartorius CP 224s, Germany)
4. Centrifuge (Universal 320r, Hettich, Germany)
5. CO\textsubscript{2} Incubator (HERA Cell 240, Heraeus, Germany)
6. Desiccators (Biologix Research Company, USA)
7. FTA 1000 drop shape instrument (First Ten Angstroms, USA)
8. Differential scanning calorimeter (Sapphire, PerkinElmer, USA)
9. Freeze dryer (FreeZone2.5, Labconco, USA)
10. FT-IR spectrophotometer (Magna-IR system 750, Nicolet Biomedical Inc., USA)
11. Hot air oven (Heraeus, Germany)
12. Hot stage microscopy (Mettler Toledo FP82HT, Switzerland)
13. Hydraulic press (Specac Inc., USA)
14. Laminar air flow cabinet (Hera Safe, Heraeus, Germany)
15. Light microscope (CX41RF, Olympus, Japan)
16. Loss on drying measurement (Sartorious Moisture balance, Germany)
17. Micro-plate reading spectrofluorimeter (EL808, Biotek, USA)
18. Magnetic stirrer and Magnetic bar (Mettler-toledo GmbH, Germany)
19. pH meter (Mettler Toledo seveneasy, Switzerland)
20. Particle size analyzer (LA-950, Horiba, Japan)
21. Powder X-Ray diffractometer (D8, Bruker, Germany)
22. Protein assay kit (Micro BCATM, Pierce Chemical Company, Rockford, IL, USA)
23. Scanning electron microscope (SEM; MX2000, Camscan, UK)
24. Shaker incubator (SL SHEL LAB, SI4-2, USA)
25. UV-Spectrophotometer (Lamda2, Perkin-Elmer, USA)
26. Spray dryer (model SD-60, Labplant, UK)
27. Transmission electron microscope (TEM; JEM-2100, Jeol, Japan)
3. Methods

3.1 Preparation of raw materials

3.1.1 Preparation of chitosan glutamate

Chitosan glutamate (CG) was prepared by dissolving the chitosan base (MW; 35, 45 and 200 kDa) in glutamic acid solution. The molar ratio of glucosamine and glutamic acid was 1:1 mole. In order to avoid excess glutamic acid, minimum amount of glutamic acid was used to give an exact clear solution. The solution was adjusted to 2000 g with distilled water to make a 1% w/w solution, and was stirred for 12 h. The solution was spray-dried (Labplant Spray Dryer, SD-06, UK) with an inlet temperature of 130°C and a spraying rate of 5 mL/min.

3.1.2 Preparation of the shellac solution

The shellac solution was prepared by dissolving shellac in ammonium hydroxide solution. The amount of ammonium hydroxide solution added was based on the calculation of the AV of the shellac according to our previous report [28]. The solutions were stirred for overnight.

3.1.3 Preparation of hydrolyzed shellac

The method for hydrolysis of shellac was studied using methods previously described in Limmatvapirat et. al [30]. Briefly, shellac was hydrolyzed by reacting with 2.0 %w/w sodium hydroxide at 30±1 °C for 0, 15, 30 and 45 min (HY 0, HY 15, HY 30, and HY 45). The hydrolyzed shellac was neutralized by 2N sulfuric acid and washed with excess water and dried overnight at room temperature of 25°C.

3.2 Characterization of raw materials

The physicochemical properties of dried powder of chitosan glutamate and hydrolyzed shellac were investigated. They were AV, Fourier transform infrared (FT-IR) spectrum, differential scanning calorimetry (DSC), TG-DTA, powder X-ray diffractometry, simultaneous XRD–DSC measurement, surface
free energy, moisture content, pH, hot-stage microscopy and morphology examination.

3.2.1 Fourier transform infrared (FT-IR) spectroscopy

Fourier transformed infrared (FT-IR) spectroscopy was used to characterize the raw materials by using KBr disc method. Each sample was dried over silica gel. The samples were pulverized, blended with KBr, and compressed for the measurement using a FT-IR spectrophotometer (Nicolet, Magna 750, USA).

3.2.2 Differential scanning calorimetry (DSC)

DSC thermograms of samples were measured by using a differential scanning calorimeter (DSC 7, Perkin-Elmer, USA). 2–4 mg of sample was accurately weighed onto a solid aluminum pan, and sealed. The measurement was performed between 25 and 300 °C under the purging of nitrogen gas at a heating rate of 10 °C/min.

3.2.3 Acid value (AV)

The AV was determined by acid-base titration method adapted from United States Pharmacopeia USP XXIII [210]. An accurate weight of 2 g of hydrolyzed shellac was dissolved in ethanol for overnight and finally adjusted to the total weight of 26 g with ethanol. The solution was centrifuged for 10 min at 4000 rpm. 26 g of filtrate was titrated with 0.1N sodium hydroxide. The end point was determined by potentiometric titration instead of using a color indicator due to the dark color of shellac. The graph between millitres of NaOH and pH was plotted and the AV was calculated from the value of midpoint of the sharp change of pH. The AV was expressed as milligram of potassium hydroxide for neutralization of free carboxyl group of shellac (Eq.1). The average of two measurements was performed (n=2).

\[
AV = \frac{(\text{ml NaOH} \times 56.11)}{(40 \times W)} \quad \text{Eq.1}
\]

Where W was the accurate weight of shellac.

3.2.4 TG-DTA

Thermogravimetry-differential thermal analysis (TG/DTA) was carried out by using a TG/DTA 8120 system (Rigaku, Tokyo Japan). Samples of 5 mg were accurately weighed into open aluminum pans under purging nitrogen gas.
The measurement was performed between 25 and 300 °C under the purging of nitrogen gas at a heating rate of 10 °C/min.

3.2.5 Powder X-ray diffractometry

X-ray diffraction patterns were measured on a D8 DISCOVER (Bruker AXS, Germany) diffractometer at 40 kV and 40 mA. The relative intensities were studied within the range of 3.7 to 26.2° and the data collection time was 180 s.

3.2.6 Simultaneous XRD–DSC measurement

The method for XRD–DSC measurement in situ was studied using a Rigaku XRD-DSCII system (Rigaku, Tokyo Japan) as previously described by E. Yonemochi et al. [211]. Briefly, 5 mg of samples were weighed into 7mm×7mm×0.25mm opened aluminum pans. The heating rate of the DSC was 1 °C/min and purging by nitrogen gas at a flow rate of 50ml/min. X-ray diffraction was then measured simultaneously, and it was operated at 50kV and 40mA.

3.2.7 Wettability and surface free energy of shellac and hydrolyzed shellac

The samples were dried by vacuum freezer at -50°C for 24 h to remove residual water. The residual water was detected before and after freeze dried process by Karl Fischer titration. The hydrolyzed shellac was then compressed into tablet. The contact angle was measured using the sessile drop technique, FTA 1000 high performance image processing (First Ten Angstroms, USA) Three different standard liquids; distilled water, formamide and ethylene glycol were used to determine the contact angle at 25 °C as shown in Figure 13. The surface free energy was calculated using Wu harmonic mean method equation as the following equations. The obtained surface free energy was the average of three calculated surface free energy (n=3):
$\gamma_s, \gamma_L$ are total surface free energy. $\gamma_{SL}$ is the solid-liquid interfacial energy

$$\gamma_s = \gamma_s^d + \gamma_s^p$$  \hspace{1cm} \text{Eq.2}$$

$$(1 + \cos \theta) \gamma_s = [4 \left( \gamma_s^d \gamma_L^d / \gamma_s^d + \gamma_L^d \right) + 4 \left( \gamma_s^p \gamma_L^p / \gamma_s^p + \gamma_L^p \right)] \hspace{1cm} \text{Eq.3}$$

where $\gamma_s, \gamma_s^p, \gamma_s^d$ are total surface free energy, polar and dispersive forces of solid, respectively. $\gamma_L, \gamma_L^p, \gamma_L^d$ are total surface free energy, polar and dispersive forces of standard liquid, respectively. $\theta$ is the contact angle between the solid and the standard liquid.

### 3.2.8 Moisture content

The shellac and hydrolyzed shellac were accurately weighed ($W_0$) and dried by loss on drying measurement ($W_1$). The weight of shellac before and after drying was calculated for the moisture content (Eq.4). All samples were performed in triplicate (n=3).

$$\text{Moisture content (\%)} = 100(W_0 - W_1) / W_0 \hspace{1cm} \text{Eq.4}$$

Where $W_0$ and $W_1$ were the constant weight before and after drying

### 3.2.9 pH

The pH of samples was measured using pH meter (Mettler Toledo seveneasy, Switzerland). The average of three measurements was performed (n=3).
3.2.10 Hot-stage microscopy

The thermal properties of shellac and hydrolyzed shellac after treatment by various times were investigated by hot-stage microscopy. The samples were heated by a hot-stage (FP82HT, Mettler Toledo, Switzerland) at the scanning speed of 1 °C/minute and observed under an optical microscope (CX41, Olympus, Japan). Changes in the morphology (melting or crystallization) were noted as a function of temperature.

3.2.11 Morphology examination

Morphological examinations of the samples were performed by Scanning electron microscope (SEM, VE-7800, KEYENCE company, Japan). Samples were mounted on metal stubs and coated with a fine gold layer under vacuum before obtaining the micrographs. The acceleration voltage used was 5 kV.

3.3 Preliminary study

3.3.1 Preparation of BSA-loaded chitosan-shellac nanoparticles (BSA-loaded CG-HY NP)

BSA-loaded CG-HY NP was prepared by ionic cross-linking between the cationic molecules of chitosan and the anionic molecules of shellac for the encapsulation of BSA as a model protein. The concentrations of CG 35 and HY 0 were in the range of 0.090% - 0.110% w/v and 0.030%-0.070% w/v, respectively. The BSA at the concentrations of 0, 1.0, 1.5 and 2.0 mg/mL was mixed with the CG 35 solution for 5 minutes and the HY 0 was then added drop-wise. The colloidal solution was further stirred for 30 min. The effect of the concentrations of CG 35 and HY 0 was investigated for the fixed concentrations of HY 0 and CG 35 at 0.050% w/v and 0.100% w/v, respectively. The BSA-loaded CG-HY NP were characterized in terms of their particle size, zeta potential, morphological examination, Fourier transformed infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC), BSA encapsulation efficiency and BSA loading efficiency.

3.3.2 Particle size, Zeta potential and morphological examination of BSA-loaded chitosan-shellac nanoparticles

The BSA-loaded CG-HY NP were characterized in terms of their particle size using the light scattering technique (Horiba, LA-950, Japan), and
the zeta potential was measured by the Zeta Plus (Brookhaven Instruments Co. USA). All measurements were carried out in triplicate (n=3). Morphological examination of the nanoparticles was performed by a transmission electron microscope (TEM, TEM-1230, JEOL, Japan). One drop of the sample was placed on a copper grid, adsorbed with a filter paper and air-dried for 20 minutes, prior to the morphology study.

3.3.3 Fourier transformed infrared (FT-IR) spectroscopy and Differential scanning calorimetry (DSC)

Fourier transformed infrared (FT-IR) spectroscopy was used to characterize the BSA-loaded CG-HY NP and to confirm the ionic cross-linking between the cationic molecules of chitosan and the anionic molecules of shellac. The nanoparticles were separated from the colloidal solution by high speed centrifugation at 11,000 rpm, 25°C for 10 min (Universal 320 R, Germany). The supernatant from the centrifugation was decanted and the precipitated nanoparticles were dried in a vacuum freezer at -50°C for 18 h. The nanoparticles were pulverized, blended with KBr, and compressed for the measurement using a FT-IR spectrophotometer (Nicolet, Magna 750, USA). DSC thermograms of samples were analyzed in the same manner as described in section 3.2.2.

3.3.4 Evaluation of BSA encapsulation and loading efficiencies

The BSA-loaded CG-HY NP was separated from the colloidal solution by high speed centrifugation at 11,000 rpm, 25°C for 10 min. The free BSA content from the supernatant was analyzed by a UV spectrophotometer (Lambda 2, Perkin-Elmer, Germany) at 550 nm using the Lowry method. Triplicate samples were analyzed and the encapsulation efficiency (EE) and loading efficiency (LE) were calculated using equations 5 and 6, respectively (n=3).

\[
%EE = \left( \frac{\text{total amount of BSA} - \text{free amount of BSA from supernatant}}{\text{total amount of BSA}} \right) \times 100 \ldots \ldots \text{Eq.5}
\]

\[
%LE = \left( \frac{\text{total amount of BSA} - \text{free amount of BSA from supernatant}}{\text{Nanoparticle weight}} \right) \times 100 \ldots \ldots \text{Eq.6}
\]
3.3.5 Evaluation of BSA release

5 mL of the BSA-loaded CG-HY NP was placed in a 50 mL bottle, and 10 mL of a 0.1M phosphate buffer solution (PBS) at pH 7.4 was added. The colloidal solution was shaken at 150 rpm, and incubated at 37 °C. At proper time intervals, a sample was removed, and the same amount of PBS was added. High speed centrifugation at 11,000 rpm and 25 °C for 10 min was conducted to separate the nanoparticles. Triplicate samples of free BSA content from the supernatant were analyzed by using the Lowry method [24] (n=3).

3.4 Preparation of BSA-loaded chitosan-hydrolyzed shellac nanoparticles (BSA-loaded CG-HY_HY NP)

BSA-loaded CG-HY NP was prepared. The concentrations of CG and HY were in the range of 0.075% - 0.175% w/v and 0.150%-0.250% w/v, respectively and the BSA concentrations were 0, 0.5, 1.0, 1.5 and 2.0 mg/mL. Instead of drop wise addition of HY, the hydrolyzed shellac solution was added into the mixture of CG and BSA. The colloidal solution was further stirred for 10 min. The effect of the concentrations of CG and HY was investigated for the fixed concentrations of HY and CG at 0.200%w/v and 0.125% w/v, respectively. BSA-loaded CG-HY NP were characterized in terms of their zeta potential, particle size, BSA encapsulation efficiency and BSA loading efficiency. The physical properties were investigated by using powder X-ray diffraction, TG-DTA and Simultaneous XRD–DSC measurement. The effects of molecular weights of CG, hydrolysis time of shellac and pH adjustment were also investigated. For the effect of pH adjustment on the nanoparticle formation, pH of CG solution was adjusted to 4, 4.5, 4.75, 5, 5.25 and 5.5 by using 0.1 N HCl or 0.1 N NaOH prior to form nanoparticle.

3.5 Preparation of enteric-coated capsules filled with freeze-dried BSA-loaded CG-HY NP

The nanoparticles were prepared following the methods described in section 3.4. After that the nanoparticles were separated from the colloidal solution by high speed centrifugation at 11,000 rpm, 25°C for 10 min (Universal 320 R, Germany). The supernatant was decanted and the precipitated nanoparticles were
dried in a vacuum freezer at -50°C for 24 h. Prior to freeze drying process trehalose or glucose at the concentration of 5, 7.5 and 10 %w/v were added as a cryoprotectant to prevent aggregation. Each sample was performed in triplicate (n=3). The dried nanoparticle was then kept in desiccators for further study. The effect of freeze drying on the physicochemical properties was compared by re-dispersion in water and was measured for particle size, zeta potential, BSA encapsulation efficiency and BSA loading efficiency as described above. The comparison was also made with the dried nanoparticles with and without the aid of cryoprotectant. The dried nanoparticles were then filled in hard gelatin capsules (size 0, Torpac Inc., Fairfield, NJ, USA) as previously described by Sonaje et al. [212]. The enteric coating of capsules was then performed by the immersion method in a methanol solution of 15% w/v Eudragit® S100 and 15% w/v L100-55 Evonik Industries, Parsippany, NJ, USA). The immersion was carried out for 15 s followed by drying at room temperature using an air-blower. The coating was done 3 times for each sample. The enteric-coated capsules filled with freeze-dried BSA-loaded chitosan shellac nanoparticles and the subsequent release profiles of BSA from the freeze-dried NPs were investigated in the distinct pH dissolution media simulating the GI conditions at 37 °C under agitation (150 rpm). The comparison was made with those without enteric coating. At particular time intervals, the free BSA content from the supernatant was analyzed by a UV spectrophotometer (Lambda 2, Perkin-Elmer, Germany) at 550 nm using the Lowry method. The triplicate samples were performed for each formulation (n=3).

3.6 Stability of nanoparticles

All formulations were investigated for their stability. The formulations were nanoparticle at various concentrations of HY, BSA and CG including hydrolysis time in dried powder with and without various concentration of cryoprotectant, and some formulations were investigated in colloidal form. Formulations were stored at ambient conditions and 4 °C in a period of 0-3 months storage. The samples were studied for particle size, pH, zeta potential, encapsulation and loading efficiency immediately after preparation, and after 3 months of storage. The re-dispersion in water was required for powder form prior to investigation. Triplicate measurement was performed for all samples (n=3). The stability of BSA at
the storage time points of 0 and 3 months was also determined by native polyacrylamide gel electrophoresis as described below:

3.6.1 *Native polyacrylamide gel electrophoresis (PAGE)*

PAGE was used to monitor conformation of BSA in nanoparticle form, effect of types of cryoprotectant, in the distinct pH dissolution media and after the various times of storage. A 12.5% w/v separating gel and a 4% w/v stacking gel were prepared at room temperature. The composition of both gels was shown in Tables 8 and 9. After the complete gelation of the separating gel (~ 60 min), the stacking gel was added. The free-form BSA was used as a control. Electrophoresis was carried out using a vertical electrophoresis apparatus and the sample gel was run at 100 V. Proteins were visualized by the Coomassie brilliant blue R-250 staining and stained gels were destained overnight in destaining solution (50% w/v methanol and 5% w/v acetic acid).

### Table 8  Composition of separating gel

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 30% acrylamide</td>
<td>4.2 mL</td>
</tr>
<tr>
<td>2 1.5 M Tris-HCl</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>3 10% ammonium persulfate</td>
<td>80 uL</td>
</tr>
<tr>
<td>4 TEMED</td>
<td>20 uL</td>
</tr>
<tr>
<td>5 DI water</td>
<td>3.45 mL</td>
</tr>
</tbody>
</table>

### Table 9  Composition of stacking gel

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 acrylamide</td>
<td>0.65 mL</td>
</tr>
<tr>
<td>2 0.5 M Tris-HCl</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>3 10% ammonium persulfate</td>
<td>25 uL</td>
</tr>
<tr>
<td>4 TEMED</td>
<td>10 uL</td>
</tr>
<tr>
<td>5 DI water</td>
<td>3 mL</td>
</tr>
</tbody>
</table>
3.7 Caco-2 cells culture protocol for use in cytotoxicity test and cellular uptake of nanoparticles

The cytotoxicity test and cellular uptake of nanoparticles was determined using the Caco-2 cells. The passage numbers of the Caco-2 cells were 23-30. Caco-2 cells were cultured using the method outlined.

3.7.1 Seeding cells on 75cm² T-flask

The supplemented EMEM* (as shown in Table 10) was warmed by heating in the water bath for 15-20 minutes at 37 °C. The Caco-2 cells were taken out from the liquid nitrogen tank and then record passage number of the cells which was taken. The cells were thawed by immersing in the water bath at 37°C. The cells at density of 200,000 cells were seeded onto 75cm² together with 14 mL of the culture medium. Cells were incubated at 37°C in humidified atmosphere of 5% CO₂ and 95% air (Forma Series II Jacketed CO2 incubator, HEPA Class 100, BIOLAB, Australia) with medium exchange after 2 days of incubation.

Table 10  Concentration of compositions of supplemented EMEM (100 mL)

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  EMEM</td>
<td>-</td>
<td>76 mL</td>
</tr>
<tr>
<td>2  Sodium pyruvate (SP)</td>
<td>-</td>
<td>1 mL</td>
</tr>
<tr>
<td>3  Non-essential amino acid (NEAA)</td>
<td>100X</td>
<td>1 mL</td>
</tr>
<tr>
<td>4  Fetal Bovine Serum (FBS)</td>
<td>20 %</td>
<td>20 mL</td>
</tr>
<tr>
<td>5  Penicillin/Streptomycin</td>
<td>10,000U/10 mg</td>
<td>1 mL</td>
</tr>
<tr>
<td>6  L-Glutamine</td>
<td>200 mM</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

3.7.2 Passaging cells

The supplemented EMEM, Phosphate Buffered Saline (PBS) pH 7.4 and Trypsin-EDTA were warmed at 37°C for 15-20 minutes in the water bath. The used medium was removed and the cells washed twice with 5 mL of PBS. 3 mL of Trypsin-EDTA was used for trypsinizing cell. The detachment of cell was observed under microscope to confirm the appearance of single cells. The cells were then incubated at 37°C for 5 minutes, as longer period of incubation would cause cell clumping. The action of Trypsin was stopped by adding 7 mL of culture medium. Cells were transferred into 50 mL Falcon’s tube and then centrifuged at 1000 x g for 2
minutes at room temperature. The supernatant was discarded and pelleted cells were resuspended with 1 mL of culture medium. The cells were seeded at density of 200,000 cells into new T-flasks with 15 mL of culture medium. Cell culture was incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, and the medium was changed every 2 days of incubation (n=1).

### 3.7.3 Cell Counting

The pelleted cells were re-suspended in 1 mL of culture medium and 10 μL of the cell suspension was sampling and mixed with 90 μL of 0.04% w/v trypan blue dye in the mixing bed of hemocytometer. 10 μL of this mixture was withdrawn and put it in each capillary of hemocytometer for cell counting. The cell was examined under a microscope (inverted light microscope, Olympus, 10x objective lens). Viable cells were marked by the yellow color whereas the dead cell showed the blue color. The percent of viable cell was calculated as the following equations 7 and 8 (n=1):

*Percentage of living cells: \[
\text{Number of cells: Living cells X 10 (dilution factor) X 2500} \quad \text{Eq.8}
\]

### 3.8 In vitro cytotoxicity

The cytotoxicity of chitosan glutamate, shellac, hydrolyzed shellac and nanoparticles were determined using the (MTT) assay. This assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of mitochondrial of living cells. Caco-2 cells (passage No. 26-33) were seeded at a density of 10,000 cells/well in 96 well-cell culture plate and pre-incubated for 48 h with 100 uL of supplemented EMEM. The used medium was removed and the cells were incubated with various concentrations of samples (CG, HY and nanoparticles). The concentrations of CG (CG 35, CG 45 and CG 200) and HY (HY 0, HY 15, HY 30, HY 45) were 0.01, 0.025, 0.05, 0.075, 0.1, and 0.25 %w/v. The concentrations of HY 30, CG35 and BSA for the preparation of nanoparticles were 0.2, 0.125 and 0.15 %w/v, respectively. These samples were diluted with HBSS at pH 6.0 and 7.4. The amount used for dilution are shown (Tables 11 and 12) and incubated for 4 and 24 h at 37 °C in 5% CO₂ and 90% relative humidity. After the specific exposure times,
sample solutions were removed and the cells were washed once with 100 μL of pre-warmed PBS with 100 μL of MTT solution (1 mg/mL in PBS) for 4 h. The medium was then removed, and the formazan crystal formed in living cells were dissolved in 100 μL DMSO per well. The colour of the cell lysate was measured at 570 nm. Dextran sulphate sodium salt (0.1% w/v) and SDS (0.1% w/v) in corresponding vehicles were used as positive and negative controls, respectively. The relative cell viability was calculated according to equation 9, and the IC50 was calculated as a sample concentration that inhibited the growth of 50% of the cells relative to non-treated control cells. Three samples of each dilution were studied (n=3).

Relative cell viability = \[\frac{OD_{570,\ sample} - OD_{570,\ blank}}{OD_{570,\ control} - OD_{570,\ blank}}\] \times 100 \text{......... Eq 9}

where OD_{570,\ sample}, OD_{570,\ control} and OD_{570,\ blank} are optical density at 570 nm of sample, control and blank solution, respectively.

Blank = DMSO; it does not has Caco-2 cells
Control = cells incubated with HBSS

3.9 Quantification of Cellular Uptake of FITC-BSA

The drug uptake study protocol was adapted from a previous research [213]. Cells were seeded at a density of 10,000 cells/well in 96 well cell culture plates and pre-incubated for 48 h with 100 uL of supplemented EMEM. After 48 h, the confluent cells were co-incubated for 4 h with FITC-BSA solution or FITC-BSA NP. The concentration of SH30, CG35 and FITC-BSA for preparing nanoparticle was 0.2, 0.125 and 0.15 %w/v, respectively. The nanoparticles and FITC-BSA solution were then diluted with HBSS according to the concentration shown in Table 13. The samples were removed and the cells after washing twice with 200 uL of ice-cold PBS were then lysed by incubation for 10 min with 0.3 mL of 0.2 % Triton X in Milli-Q water. The cell lysate were measured for fluorescence and protein content using the plate reader (at λ_{ex}: 490 nm and λ_{em}: 525 nm.) and protein assay kit (Micro BCATM, Pierce Chemical Company, Rockford, IL). The cellular uptake was expressed as the amount expressed as % of initial loading of FITC-BSA solution or
FITC-BSA NP (μg) associated with unit amount of cellular protein as shown in the following Eq 10 and Eq 11, respectively. The plate reader was calibrated with standard solutions of FITC-BSA solution or FITC-BSA NP in cell lysate (1.0 * 10⁵ cells/mL in 0.2 % Triton X) at concentrations of 0-0.40 mg/mL of lysate solution (n=3).

Table 11  Concentrations of CG or HY and the amount used of HBSS

<table>
<thead>
<tr>
<th>*Conc. of CG/HY (% w/v) (n=3)</th>
<th>Volume of sample (μL)</th>
<th>Volume of HBSS (μL)</th>
<th>Total volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*0.010</td>
<td>2</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>*0.025</td>
<td>5</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>*0.050</td>
<td>10</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>*0.075</td>
<td>15</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>*0.100</td>
<td>20</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>*0.250</td>
<td>30</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

*Stock solution of CG/HY = 0.5 % w/v

Table 12  Concentrations of Nanoparticle dispersion and the amount used of HBSS

<table>
<thead>
<tr>
<th>Conc. of Nanoparticle dispersion (% v/v) (n=3)</th>
<th>Volume of sample (μL)</th>
<th>Volume of HBSS (μL)</th>
<th>Total volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>80</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 13  Prepared concentrations of free FITC-BSA and loaded FITC-BSA NP

<table>
<thead>
<tr>
<th>Conc. of sample (% v/v) (n=3)</th>
<th>Volume of sample (μL)</th>
<th>Volume of HBSS (μL)</th>
<th>Total volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>80</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
% initial loading = \frac{\text{Amount of FITC-BSA solution or FITC-BSA NP (\(\mu\)g) \times 100}}{\text{Amount of initial FITC-BSA}} \quad \text{Eq10}

\text{Amount of cellular uptake} = \frac{\text{Amount of FITC-BSA solution or FITC-BSA NP (\(\mu\)g)}}{\text{Amount of cellular protein (\(\mu\)g)}} \quad \text{Eq11}

\textbf{3.10 Statistical analysis}

Data was expressed as means ± standard deviation (SD) of three determinations (n=3). The statistical significance analysis was carried out using analysis of variance (ANOVA) at the 0.05 significant levels.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Characterization of raw materials

4.1.1 Morphology examination

The physical morphology of native shellac and hydrolyzed shellac at various times is shown in Figure 14. After grinding, the fine powder of native shellac was observed. The dark color was seen after hydrolysis process. The physical color of the HY 15 shellac quite differed from the HY 30 and HY 45. The longer time of hydrolysis, the darker brown color and stickier than native shellac was obtained. Owing to the stickiness, the maximum time could be performed in this study was 45 minutes further hydrolysis could not be carried out. Hence, the hydrolysis time of shellac with sodium hydroxide had an influence on the physical morphology of shellac.

Figure 14  Physical characteristic of hydrolyzed shellac at various times; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45, respectively
SEM photograph was used to support the morphology of each hydrolysis time as shown in Figure 15. The irregular shape and rough surface were displayed on all hydrolyzed shellacs.

Figure 15  SEM photographs of hydrolyzed shellac at various times; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45, respectively

Figure 16  SEM photographs of (A) chitosan base and (B) chitosan glutamate
Figure 16 shows the morphology of chitosan base and chitosan glutamate. The irregular shape and rough surface were displayed on the chitosan base whereas chitosan glutamate showed the spherical shape and smooth surface. The particle size range of chitosan glutamate was around 2-10 μm.

4.1.2 Hot-stage microscopy

The hot-stage microscopy was used to investigate the change of native shellac and hydrolyzed shellac under an alteration of temperature and screen for melting temperature. Hot-stage microscopy is the combination of microscopy and thermal analysis for the physical characterization of materials under the change in temperature and time. It is a very useful technique to rapidly distinguish between the crystalline and amorphous form of materials [214]. The degree of temperature is structure dependent. The stronger the structure, the higher temperature is required to break the bond of a material. The start of melting temperature indicates by the beginning of transparency of sample. The HY 0 showed the melting temperature approximately at 84 °C as shown in Figure 17 while the melting temperature of each hydrolyzed shellac decreased with the increase in hydrolysis time. The start of melting temperature of HY 15, HY 30 and HY 45 were around 75 °C, 70 °C and 60 °C as shown in Figures 18, 19, and 20, respectively. The decrease in the melting temperature with the prolonged hydrolysis time was a result of breaking of the ester bonds of shellac during the hydrolysis process. The longer hydrolysis time the higher extent of breaking of the ester bonds was obtained, contributing to the lower strength of the structure of polymer network [30]. In this study, the melting temperature of native and all hydrolyzed shellacs were around 60-120 °C which was in agreement with the work of Limmatvapirat et. al. [30].
Figure 17  Hot-stage microscopic images of HY 0 at (A) 50 °C, (B) 80 °C, (C) 84°C , (D) 88 °C, (E) 90 °C and (F) 95 °C, respectively

Figure 18  Hot-stage microscopic images of HY 15 at (A) 50 °C, (B) 70 °C, (C) 75°C , (D) 80°C , (E) 85 °C and (F) 90 °C, respectively
Figure 19  Hot-stage microscopic images of HY 30 at (A) 50 °C, (B) 65 °C, (C) 70°C, (D) 76°C, (E) 80 °C and (F) 90 °C, respectively

Figure 20  Hot-stage microscopic images of HY 45 at (A) 50 °C, (B) 55 °C, (C) 60 °C, (D) 65 °C, (E) 70 °C and (F) 75 °C, respectively
Table 14 Acid value, % water content and % insoluble solid content of HY 0, HY 15, HY 30 and HY 45

<table>
<thead>
<tr>
<th>Hydrolysis time (minutes)</th>
<th>Acid value ± SD</th>
<th>% Water content ± SD</th>
<th>% Insoluble solid ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>77.98 ± 1.78</td>
<td>1.49 ± 0.02</td>
<td>1.26 ± 0.16</td>
</tr>
<tr>
<td>15</td>
<td>97.70 ± 1.21</td>
<td>5.36 ± 0.77</td>
<td>3.21 ± 0.16</td>
</tr>
<tr>
<td>30</td>
<td>106.93 ± 0.82</td>
<td>13.96 ± 0.16</td>
<td>1.89 ± 0.11</td>
</tr>
<tr>
<td>45</td>
<td>109.65 ± 0.72</td>
<td>11.12 ± 2.30</td>
<td>2.35 ± 0.12</td>
</tr>
</tbody>
</table>

4.1.3 Acid value, % water content and % insoluble solid of native shellac and hydrolyzed shellac

The effect of alkaline treatment on the AV, % water content and % insoluble solid of shellac are shown in Table 14. The % insoluble solid content did not significantly differ, and was around 1-3%. The longer hydrolysis time caused the higher breaking of the esters bond of shellac giving the free carboxyl groups indicating by the increase in the AV [30]. The higher carboxyl groups led to the increase in water content due to the higher affinity to water. The breaking of ester bonds was in accordance with the melting temperature. In addition, the different hydrolysis rate was also observed (Table 14). The hydrolysis of polyester chain took place to a greater extent at the initial 15 min of hydrolysis and then was reduced at a longer period. Two possible assumptions for this different rate of hydrolysis were explained. The first assumption could be due to different bond strength or steric hindrance of various ester bonds of shellac. Shellac is composed of polyester and single ester with polyhydroxy polybasic acids such as aleuritic acid, jalaric acid, and laccijalaric acid. Different types of ester bond showed different strengths attributing to different rates of hydrolysis. [30]. The second assumption resulted from varying structure of crystallinity. The high hydrolysis rate would expect to occur at the amorphous region, the loose part, whereas the crystalline region had the lower rate. However, the second assumption proved to be incorrect since clear diffraction peak due to crystalline part was not observed in the powder X-ray diffractogram of native shellac. In the case of shellac, different ester bonds should be a more possible explanation for different hydrolysis rate [30]. The rate of change in % water content was in a similar manner; ie, the initial stage showed the higher hydrolysis rate and
was reduced at the longer time. Our work was in agreement with the previous study which reported the higher extent of change in several parameters such as water permeability coefficient, mechanical properties and solubility etc at the initial 15 min of hydrolysis time [215].

Table 15 Water content of HY 0, HY 15, HY 30 and HY 45; before and after freeze drying

<table>
<thead>
<tr>
<th>Hydrolysis time (minutes)</th>
<th>% Water content Before Freeze dried</th>
<th>% Water content After Freeze dried</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.49 ± 0.002</td>
<td>0.95 ± 0.000</td>
</tr>
<tr>
<td>15</td>
<td>5.36 ± 0.773</td>
<td>0.55 ± 0.242</td>
</tr>
<tr>
<td>30</td>
<td>13.96 ± 0.160</td>
<td>1.88 ± 0.300</td>
</tr>
<tr>
<td>45</td>
<td>11.12 ± 2.300</td>
<td>0.24 ± 0.353</td>
</tr>
</tbody>
</table>

4.1.4 Wettability and surface free energy of shellac and hydrolyzed shellac

The sessile drop technique was used for determining surface free energy and contact angle of shellac and hydrolyzed shellac. The water content has the effect on the characteristic nature of solid surface. Hence, the residual water content was removed prior to the contact angle measurement. Table 15 shows the water content of hydrolyzed shellac before and after the process of freeze drying. The result demonstrated that the water content could be removed from the solid. Figure 21A displays the effect of hydrolysis time on the contact angle of hydrolyzed shellac at various times. The effect of hydrolysis time tended to reduce the contact angle however the slight difference was shown at 30 and 45 minutes of hydrolysis time. The decrease in the contact angle during the hydrolysis process could be the result of the breaking of the ester bonds of shellac giving the free carboxyl groups as previous report [1]. The higher free carboxyl groups had a high affinity to water, so the angle was decreased. The higher decrease in the contact angle at the beginning of hydrolysis time (0-30 min) was in agreement with the higher change in AV while at 30 and 45 minutes of hydrolysis time did not show the difference.
Figure 21  Surface characteristic of HY 0, HY 15, HY 30 and HY 45: (A) contact angle, (B) component of surface free energy and (C) total surface free energy
Figures 21B and 21C show the effect of hydrolysis time on dispersive and polar forces and total surface free energy. The hydrolysis time tended to decrease in the dispersive force and the increase in the polar force and total surface free energy. This result suggested that the hydrolysis process caused the breaking of the ester bonds of shellac giving the higher free carboxyl group indicating by the higher polar force and total surface free energy [216]. This proved that the hydrolysis process attributed to the hydrophilic nature of shellac. The prolong hydrolysis time gave the higher hydrophilic surface and was in agreement with the higher water content and AV. The higher rate of change in polar force and total surface free energy at the beginning of hydrolysis time (0-30 min) was in accordance with the higher change of other parameters while at 30 and 45 minutes of hydrolysis time did not show the difference.

Figure 22  DTA thermograms of; (A) HY 0, (B) HY 15 , (C) HY 30  and (D) HY 45
4.1.5 DTA of shellac and hydrolyzed shellac

The DTA thermograms of shellac and hydrolyzed shellac are shown in Figure 22. At the initial temperature, the substances presented the endothermic peaks around 40-100 °C attributing to the elimination of absorbed water. The melting endothermic peak of all hydrolyzed shellacs also displayed at these range of temperatures [28, 30]. Unlike the hot-stage microscopy, the DTA thermogram could not be used to determine the beginning of melting temperature of all hydrolyzed shellacs owing to the amorphous region and the broad peak in the shellac structure.

4.1.6 Powder X-ray diffractometry

The X-ray diffraction pattern of the samples is shown in Figure 23. The HY 0, HY 15, HY 30 and HY 45 showed slight sharp peak at around 21.6°. The hydrolysis process did not give any change differently among all types of hydrolyzed shellacs. All hydrolyzed shellacs showed similar pattern. The longer time (HY 30 and HY 45) of hydrolysis exhibited the flat pattern at 8-10° while 0 and HY 15 showed a broad peak.

Figure 23  XRD pattern of ; (A) HY 0, (B) HY 15 , (C) HY 30  and (D) HY 45
4.1.7 FTIR spectra of shellac and hydrolyzed shellac

The FT-IR spectra of HY 0, HY 15, HY 30 and HY 45 are shown in Figure 24. All spectra displayed a broad band at 3,000-3,600 cm\(^{-1}\), indicating the overlap of OH and NH stretchings at the same region [15, 29]. The characteristic peaks were the symmetric and asymmetric carbonyl stretchings of the carboxylate of shellac at 1,559 cm\(^{-1}\) and 1,386 cm\(^{-1}\), respectively [28, 29]. In addition, the bands at 1,716 cm\(^{-1}\) and 1,255 cm\(^{-1}\) were assigned to the carbonyl stretching vibration and C–O stretching band, respectively [29]. All the hydrolyzed shellacs displayed similar spectra; hence, the hydrolysis time did not affect the main structure of shellac.

Figure 24  FTIR spectra of hydrolyzed shellac at various times; (A) HY 0, (B) HY 15, (C) HY 30 and (D) HY 45.
4.1.8 Simultaneous XRD–DSC measurement of native shellac and hydrolyzed shellac

Parallel measurements of separate specimens using DSC and XRD, and a combination of both results are one of the popular methods for studying thermal reactions of solids[217]. Simultaneous XRD and DSC measurements can overcome problems arising from separate measurements of XRD and DSC such as specimen inhomogeneity, the difference in the temperature distribution, the accuracy of the temperature measurement, the effect of specimen mass and self-generated atmosphere on the reaction.[218] Simultaneous XRD and DSC measurement was used to prove the result of DTA and XRD of HY 0, HY 15, HY 30 and HY 45 as shown in Figures 25-28. The characteristic peak of native shellac and hydrolyzed shellac were displayed at 21.6° that exhibited at the same position as X-ray measurement. The characteristic peak at 21.6° of all hydrolyzed shellacs was disappeared at around 40-65 °C. The results could be confirmed that there was the elimination of adsorbed water and the melting temperature [28].

Figure 25  XRD-DSC data of HY 0
Figure 26  XRD-DSC data of HY 15

Figure 27  XRD-DSC data of HY 30
Figure 28  XRD-DSC data of HY 45

Figure 29 shows the simultaneous XRD-DSC data of the CH. The two crystalline peaks of CH at around 10.4° and 19.9° were disappeared when the temperature increased from 260 °C to 300 °C. The exothermic peak of decomposed chitosan was also shown around 280 °C. From these results suggested that the complete decomposition of chitosan by de-polymerization of chains including decomposition of pyranose rings via dehydration and deamination and eventually ring-opening reaction might be occurred at these temperatures range [219]. The characteristic peak of CG at around 19.9° as shown in Figure 30 was disappeared at the same range of temperature of CH. The diffraction pattern of BSA is shown in Figure 31. The broad characteristic peak of BSA at 9.5° was disappeared and the decomposition of BSA was taken, at temperature of 200-250 °C. The result was in accordance with the other report [220].
Figure 29  XRD-DSC data of chitosan base (CH)

Figure 30  XRD-DSC data of chitosan glutamate (CG)
4.2 Preliminary study

4.2.1 Physicochemical characterizations of BSA-loaded CG-HY NP

The two biopolymers were used to prepare nanoparticles for protein delivery systems by the ionic cross-linking technique. Depending on the
concentrations of CG 35, HY 0 and BSA, there were three physical states; nanoparticle, aggregation and solution as shown in Figure 32 [9, 221].

Table 16  Effect of different concentrations of CG 35, HY 0 and BSA on particle size and zeta potential of BSA-loaded CG-HY NP (mean±S.D., n=3)

<table>
<thead>
<tr>
<th></th>
<th>Chitosan glutamate concentrations (% w/v)</th>
<th>With BSA (mg/ml)</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
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<td>2.0</td>
<td>1.0</td>
</tr>
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<td>0.090</td>
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<td>280.0±18.2</td>
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</tr>
<tr>
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<tr>
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<td>Sol</td>
<td>144.0±4.2</td>
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</tr>
<tr>
<td>**Shellac concentrations (% w/v)</td>
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<td></td>
</tr>
<tr>
<td>0.030</td>
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</tr>
<tr>
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</tr>
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<td>106.3±1.5</td>
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</tbody>
</table>

Ag = Aggregation state
Sol = Solution state
* The concentration of HY 0 was fixed at 0.050 % w/v
** The concentration of CG 35 was fixed at 0.100 % w/v

Table 16 shows the effect of various concentrations of CG 35, HY 0 and BSA on their particle sizes and zeta potential. The particle size of the colloid was in the nanometer size range of 100-300 nm. For 1 mg/mL of BSA, at various concentrations of HY 0, the nanoparticles could not be formed, and only a clear solution was obtained. This proved that the formation was concentration dependence. The zeta potentials of all nanoparticles displayed a positive charge. The positive charge of the nanoparticles was attributed to the unoccupied amine groups of the
chitosan. The increase in the concentration of CG 35 at the fixed concentration of HY 0, the higher zeta potential was obtained [18] because of the increase in the positive charge from the amine groups of chitosan. However, the increase in the negative charge of HY 0 did not result in the reduction in the zeta potential, which was not in agreement with the use of tripolyphosphate (TPP) as polyanion to form nanoparticles [17].

The increase in the zeta potential with the increase in the concentration of HY 0 was attributed to the increase in the unoccupied amine group of CG 35 due to the competitiveness of deprotonated carboxylic groups between HY 0 and BSA and the large molecule of HY 0. These were attributed to the difficulty in interaction with amine group of chitosan. The addition of BSA caused the reduction in the zeta potential and the higher BSA concentrations showed the higher decrease in the zeta potential [222]. The result proved that the BSA showed a negative charge as the pH being studied was higher than the pI value (pI=4.7, the pH under this condition was approximately 5.5). There was the electrostatic interaction between NH3+ from the chitosan and the negative charge from the BSA. The result was in agreement with the reports of Boonsongrit et al and Chen et.al [17, 97]. They found that the zeta potential decreased with the increase in the BSA concentration resulting from the increased negative charge of BSA.
Figure 33 shows the state of interaction of HY, CG and BSA. They were the formation of the nanoparticles (A), aggregation (B) and solution (C). Figure 33 A shows the formation of the nanoparticles due to the ionic interaction between the positive charge of CG and the negative charge of BSA and HY. The effect of other ions from glutamate and ammonium ions had little influence on the state of interaction owing to the exact calculation of the amount of glutamic acid and ammonium hydroxide solution used for the preparation of chitosan and shellac solutions as described above. The optimum concentrations of CG 35, HY 0 and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles. The charge of the nanoparticles attributing to the stability of colloids was reported [11, 20]. The high positive charge of the nanoparticles gave the high repulsive force, causing the individual nanoparticles. In this study, the zeta potential of the nanoparticles was in the range of 14-37 as shown in Table 16. The reduction in the zeta potential of the nanoparticles caused the nanoparticles to come closer until it was aggregated. The result was in agreement with our previous study which reported the aggregation when the zeta potential was reduced [222]. In addition, the pH value played a role on the formation of the particles as previously described [14, 18, 20]. The increase in the pH led to the reduction in the degree of protonation of the chitosan, and hence the decrease in the zeta potential. This caused the decrease in the electrostatic repulsion force between the particles leading to the aggregated particles as shown in Figure 33B [18, 20].
Figure 33 Proposed schematic representation of ionic interaction of Nanoparticulate formation (A), Aggregation (B) and Solution state (C), respectively
The aggregation was reported and was in accordance with the increase in the pH with the increase in the BSA from 1.5 mg to 2 mg/mL; at 0.090-0.095 %w/v concentration of CG 35. Figure 33C shows the solution state indicating that each molecule of CG, HY and BSA was apart. This could be due to the greater repulsive force than the attractive force at the lower BSA concentration. This caused the higher unoccupied amine group of chitosan and the higher zeta potential and hence the solution state was formed.

![Figure 34 TEM photographs of BSA-loaded CG-HY NP (CG 35 0.100 %w/v, HY 0 0.050 %w/v and BSA 1.50 mg/mL)](image)

In order to confirm the morphological characteristic of the BSA-loaded CG-HY NP, TEM was used as shown in Figure 34. The spherical shape of the nanoparticles was observed. This result could be confirmed that the nanoparticle was formed by using CG 35 and HY 0 for the encapsulation of BSA.
The FT-IR spectra of HY 0, BSA, CG 35, BSA-loaded CG-HY NP and physical mixture (PM) are shown in Figure 35. All spectra displayed a broad band at 3,000-3,600 cm⁻¹, indicating the overlap of OH and NH stretchings in the same region [15, 223]. In this region, CG 35 and PM were shown in the same peak at 3,442 cm⁻¹. In addition, this result was in line with the previous study as the peak of the nanoparticles was shifted to 3,312 cm⁻¹, indicating the hydrogen bonding formation [25]. In addition, the FT-IR spectrum of CG showed a broad peak due to the amide stretching at 1,633 cm⁻¹ and a strong protonated amino peak at 1564 cm⁻¹ [14]. The spectrum of the nanoparticles was shifted from 1,633 cm⁻¹ to 1,660 cm⁻¹ and the sharp peak was found due to the ionic interaction between the amine group of chitosan and the hydroxyl group of BSA, supporting the formation of the nanoparticles [177]. The result of sharp peak formed was in agreement with our previous study using TPP as
polyanion[16] In addition, the FTIR bands at 1,660 cm\(^{-1}\) and 1,539 cm\(^{-1}\) of the nanoparticles were assigned to the amide I and amide II bands respectively, which are the characteristic of protein spectra [14], supporting the presence of BSA within the nanoparticles. The amide I and amide II resulted from the amide bonds that linked the amino acids. The absorption associated with the amide I band led to stretching vibrations of the C=O bond of the amide, absorption associated with the amide II band led primarily to bending vibrations of the N—H bond [224]. The bands at 1,559 cm\(^{-1}\) and 1,386 cm\(^{-1}\) were also assigned to the symmetric and asymmetric carbonyl stretchings of the carboxylate of Hy 0, respectively [28]. This was slightly shifted for the spectrum of the nanoparticles at 1,394 cm\(^{-1}\), and the peak at 1,559 cm\(^{-1}\) of HY 0 was hardly visible for the spectrum of the nanoparticles. However, the peak at 1,255 cm\(^{-1}\) of HY 0 [29] associated with the carbonyl stretching could also be observed in the spectrum of the PM while this peak of the nanoparticles was shifted to 1,249 cm\(^{-1}\), indicating that HY 0 was part of the formation of the nanoparticles.

To confirm the result of the formation of protein-loaded nanoparticles, DSC was used to confirm the formation of protein-loaded nanoparticles. The DSC thermograms of HY 0, BSA, CG 35, BSA-loaded CG-HY NP and PM are shown in Figure 36. The DSC thermograms of all substances showed the endothermic peaks around 40-120 °C which were associated with the loss of water. The CG 35 showed the endothermic peak at 170.5 °C, indicating the glutamate of chitosan [225]. The PM was reported at the same peak but it was not found for the nanoparticles. In addition, the thermogram of BSA showed the decomposition peak at 219.2 °C while the peak of the nanoparticles shifted to 223.1 °C [14, 220] as a result of the interaction between each material for the formation of nanoparticle. Therefore, it proved that the formation of nanoparticles could be achieved by the application of two natural oppositely charged polymers for the encapsulation of BSA.
4.2.2 Evaluation of BSA encapsulation and loading efficiencies

Table 17 shows the effect of the concentrations of CG 35, HY 0 and BSA on EE and LE of BSA-loaded CG-HY NP. The EE and LE were in the range of 11-67% and 7-45 %, respectively. The increase in the concentrations of CG 35 resulted in the significant ($p<0.05$) decrease in the EE and LE, which was in an agreement with the other report [21]. This could be the result of the increase in the viscosity which was correlated with the fact that the increase in the concentrations made it difficult for the encapsulation to take place by averting the BSA molecular movement around the chitosan molecule chain [21, 226]. The decrease in EE and LE was consistent with the increase in the zeta potential indicating the higher unoccupied amine of CG 35 with the increase in CG 35. The higher amount of BSA had a tendency to increase in the EE and LE which was similar to the work of Gan and Wang [21] whereas the opposite effect was reported by other studies [25, 221].
Table 17 Effect of different concentrations of CG 35, HY 0 and BSA on encapsulation efficiency (EE) and loading efficiency (LE) of BSA-loaded CG-HY NP (mean±S.D., n=3)

<table>
<thead>
<tr>
<th>*Chitosan glutamate concentrations (w/v)</th>
<th>With BSA (mg/ml)</th>
<th>Encapsulation efficiency (%)</th>
<th>Loading efficiency (%)</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<td>21.2±1.1</td>
<td>49.2±1.0</td>
<td>Ag</td>
</tr>
<tr>
<td>0.100</td>
<td>Sol</td>
<td>22.8±0.5</td>
<td>57.6±0.2</td>
</tr>
<tr>
<td>0.105</td>
<td>Sol</td>
<td>13.9±0.5</td>
<td>37.6±3.1</td>
</tr>
<tr>
<td>0.110</td>
<td>Sol</td>
<td>13.5±0.7</td>
<td>20.4±0.3</td>
</tr>
<tr>
<td>**Shellac concentrations (w/v)</td>
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<tr>
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<td>0.040</td>
<td>Sol</td>
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<td>22.2±0.1</td>
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<td>Sol</td>
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<td>57.6±0.2</td>
</tr>
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<tr>
<td>0.070</td>
<td>Sol</td>
<td>11.9±0.2</td>
<td>27.4±0.7</td>
</tr>
</tbody>
</table>

Ag = Aggregation state  
Sol = Solution state  
* The concentration of HY 0 was fixed at 0.050 % w/v  
** The concentration of CG 35 was fixed at 0.100 % w/v

The higher EE and LE as the increase in BSA was also in accordance with the reduction in the zeta potential confirming that there was an interaction between the positive charge of CG 35 and the negative charge of BSA. The result of varying concentration of HY 0 on the change in EE and LE at the concentration of 1.5 mg/mL BSA showed similar result to varying concentration of CG 35, and was related to the change in the zeta potential. The decrease in EE and LE was significant ($p<0.05$) with the increase in HY 0. Nevertheless, at the concentration of 2.0 mg/mL,
the reduction in EE and LE did not change in the same manner as the concentration of 1.5 mg/mL, and the highest EE was obtained for the HY 0 concentration of 0.050%. The EE and LE increased from 22.2% to 57.6% and 16.2% to 41.4%, respectively when HY 0 increased from 0.040 %w/v to 0.050 %w/v. But, the increase in concentration of HY 0 from 0.050 %w/v to 0.070 %w/v, the EE and LE decreased from 57.6% to 27.4% and 41.4% to 19.3%, respectively. Therefore, it could be concluded that the formation of BSA-loaded nanoparticles was only possible for some specific concentrations of the positively charged CG 35 and the negatively charged HY 0. The optimum concentration of CG 35, HY 0 and BSA was 0.100 %w/v, 0.050 %w/v and 2.0 mg/mL, respectively due to the highest EE and LE.

Figure 37  The release profile of BSA-loaded CG-HY NP at different concentrations of CG 35 and HY 0 in phosphate buffer solution at pH 7.4, 37 °C, (mean ± SD, n = 3)
4.2.3 Evaluation of BSA release

Release profiles of many reports have exhibited, initially, the burst release of drugs or proteins from the micro or nanoparticles followed by a slow release [24, 25, 221, 223]. The mechanism of the release involved two different states; the fast release from the location of the drug on the surface led to the immediate release when it was exposed to the dissolution medium. The next step was the diffusion through the nanoparticles which was the predominant release mechanism [223]. The amount of release was controlled by the EE and the mechanism of encapsulation as presented in Figure 37. The nanoparticles showed the immediate release of BSA from the matrix of all systems, and the amount of release was between 64.3-78.1% significantly depending on the concentrations of CG 35 ($p<0.05$). After 30 minutes, a gradual and slow release was reported. The immediate release was due to the part of adsorbed BSA at the surface and the competitiveness between phosphate ion and the anion of BSA at the binding sites [26, 109]. The CG 35 concentrations affected the total release of BSA from the nanoparticles. It decreased from 87.0 to 73.4% with the increasing CG 35 concentration from 0.095 to 0.105% w/v ($p<0.05$), which was in agreement with the work of Gan and Wang [21] however, it was contrast to the report of Xu and Du [25]. The lowest release was found for 0.105%w/v CG 35 as a result of the lowest encapsulation. The higher levels of encapsulated BSA tended to have an initially higher diffusion rate contributing to a higher driving gradient. In addition, the lower release was in accordance with the larger size. The larger size at 0.105% w/v CG 35 attributed to the larger diffusion path length for the BSA and the lower contact surface area of the larger particles with the dissolution medium [13]. Hence, the release of 0.105 %w/v CG 35 was lower as a result of the larger particle size and the lower EE. The cumulative amounts of BSA released from the various concentrations of HY 0 were around 85-87%w/v, the release did not differ significantly ($p>0.05$), and showed the burst release initially. The release of BSA at 0.040 %w/v HY 0 was lower than the others ($p<0.05$), due to the lower EE and the larger size. The cumulative amount of BSA did not release completely due to the entrapment of BSA within the matrix of the nanoparticles, which was in agreement with the other studies [21, 26]. In addition, the extended release over a longer time period until 48 hours was performed; the result did not
significantly differ from the result of 24 hours. (Data was not shown) Therefore, it could be concluded that the release of BSA from the particulate systems was dependent on the size and the EE.

4.3 Characterizations of BSA-loaded chitosan-hydrolyzed shellac nanoparticles (BSA-loaded CG-HY_HY NP)

![SEM photograph of BSA-loaded CG-HY_HY NP](image)

Figure 38  SEM photographs of BSA-loaded CG-HY_HY NP; of CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL

In preliminary study, CG 35 and HY 0 could be used to form nanoparticles by polyelectrolyte complex between positively charged CG 35 and negatively charged HY0 [227]. However, owing to the large structure and low amount of anionic of native shellac attributed to the difficulty in interaction with positive charge of CG and hence the narrow range of concentration could be used. The potential of using hydrolyzed shellac at various times for the complex formation with CG for loading BSA was then carried out. Hydrolyzed shellac has a higher AV than native shellac indicating that it possesses a higher amount of negative charge on the molecular structure. In addition, it has a lower molecular weight than native shellac as clearly described by Limmatvapirat [30]. Hence, it should be easier to form complex with CG for BSA loading than native shellac. In this study, HY 30 was used for the
possibility of forming nanoparticle due to the higher in AV than HY 0, HY 15 and lower insoluble solid than HY 15, HY 45. In addition, HY 30 was less sticky than HY 45.

SEM was used to investigate morphological characteristic of the nanoparticle as shown in Figure 38. The nearly spherical shape was displayed. The particle size was lower than 1 μm, and it was correlated with using the light scattering method to detect the particle which was described in further section.

Figure 39 DTA thermograms of (A) HY 30, (B) CH 35, (C) CG 35, (D) BSA, (E) BSA-loaded chitosan-shellac nanoparticles of CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL, and (F) CG 35/ HY 30/BSA physical mixture 1:1:1

DTA and XRD were used to characterize BSA-loaded CG-HY_HY NP as shown in Figures 39 and 40. The DTA thermograms of HY30, CH 35, CG 35, BSA, nanoparticles and PM are shown in Figure 39. The initial time, the substances presented the endothermic peaks around 40-100 °C attributing to the elimination of
absorbed water and the melting endothermic peak of HY30 also displayed at the same temperature around 50 °C [28, 228]. The exothermic peak of CH 35 appeared at around 310 °C caused by de-polymerization of chitosan chains including decomposition of pyranose rings via dehydration and deamination and eventually ring-opening reaction [219]. However, the decomposition peak of CG 35 significantly shifted to around 264°C and the peak height was lower than CH 35 due to the decrease in the crystalline form of CG 35. The endothermic peak of CG 35 appeared at 173 °C [225] indicating glutamate of chitosan which was presence at the same endothermic peak of PM, but it could not be seen in the nanoparticle. The thermogram of BSA showed the decomposition peak at 217.2 °C while the peak of nanoparticle slightly shifted to 215.9 °C due to the formation of nanoparticles [220, 229, 230].

Figure 40  XRD pattern of (A) HY 30 , (B) CH 35, (C) CG 35, (D) BSA, (E) BSA-loaded CG-HY_HY NP of CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL, and (F) CG 35/ HY 30/BSA physical mixture 1:1:1
The X-ray diffraction patterns of the samples are shown in Figure 40. The HY 30 showed slightly sharp peak at around 21.6°. The CH 35 exhibited two peaks at around 10.4° and 19.9° which indicated the crystal forms I and II, respectively [231, 232]. The peak of CG 35 at around 10.4 disappeared and the peak at 19.9° became broader than the CH 35 which may be due to the decrease in the intermolecular hydrogen bonding in the chitosan [231]. Hence, the CG 35 displayed a poor crystallinity compared with the CH 35. The broad characteristic peaks of BSA are shown at around 9.5° and 21.0° [233]. Similar pattern of X-ray diffraction of nanoparticle and PM were reported indicated the presence of BSA in the nanoparticles. However, this method could predict the presence of BSA, the formation of nanoparticle by the interaction of each material could not be clarified.

Simultaneous XRD and DSC measurement was used to prove the result of DTA and XRD of each material and the formation of nanoparticle. The formation of nanoparticle was observed by the shifting of the decomposition temperature of the nanoparticle comparing with the disappearance of the decomposition temperature of active ingredient at the characteristic peak from XRD. The characteristic peak of nanoparticle at around 9.5° was disappeared at temperature in the range of 180-230°C which was correlated with the decomposition of BSA at 220°C [14, 220] as shown in Figure 41. The range of decomposition temperature of the nanoparticle did not significantly differ from decomposition temperature range of BSA as shown in Figure 31. The result suggested that it was difficult to prove the formation of nanoparticle by using simultaneous XRD and DSC measurement because it was not clear to observe the difference in decomposition temperature range between the nanoparticle and BSA. However, it was still potentially useful for characterization of chitosan, shellac or other biopolymers on the decomposition temperature or change of molecular structure. In addition, XRD and DSC measurement could be proved the formation of NP by the application of CG 35 and HY 30 that was used instead of HY 0 to form NP for encapsulation of BSA. The various factors such as molecular weights of CG, concentrations of CG, HY 30 and BSA, hydrolysis times of HY and pH of solution on the formation of BSA-loaded CG HY_HY NP will be discussed in further section.
4.4 Various factors on the formation of BSA-loaded CG HY_HY NP

4.4.1.1 Effect of molecular weight of CG on pH, zeta potential and particle size of BSA-loaded CG-HY_HY NP

BSA-loaded CG-HY_HY NP could be prepared by two biopolymers with ionic- cross linking method as described in preliminary section. Table 18 shows the the effect of molecular weight of CG and concentration of BSA on the formation of nanoparticles. The nanoparticle could be formed for all concentrations of BSA for CG 45 while for CG 35, the higher BSA (1mg/ml) the aggregation was noted. This was the result of the longer chain of high molecular weight attributing to the increased viscosity of circumstance such that the particles could not come closer including the result of the higher entanglement of structure of CG 45 than CG 35 resulting in the difficulty in the interaction with BSA [20, 24, 25]. In addition, the aggregation was the result of the reduction in repulsive force when the
higher amount of negatively charged BSA was incorporated into the lower molecular weight of positively charged CG. Hence, the particles became closer and aggregation occurred.

Table 18 Effect of different molecular weight of CG and concentrations of BSA on the formation of nanoparticles and aggregation state (HY 30 0.200 %w/v, CG 0.100 %w/v)

<table>
<thead>
<tr>
<th>BSA (mg/mL)</th>
<th>MW. Of CG</th>
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<td></td>
<td>CG 35</td>
</tr>
<tr>
<td>0</td>
<td>NP</td>
</tr>
<tr>
<td>0.5</td>
<td>NP</td>
</tr>
<tr>
<td>1.0</td>
<td>Ag</td>
</tr>
<tr>
<td>1.5</td>
<td>Ag</td>
</tr>
<tr>
<td>2.0</td>
<td>Ag</td>
</tr>
</tbody>
</table>

NP = Nanoparticle formation  
Ag = Aggregation state

The effect of molecular weight of chitosan glutamate and concentration of BSA on pH, zeta potential and particle size are shown in Figure 42. The concentration of BSA at 0.5 and 1 mg/mL were chosen in this study because the higher amount of BSA leading to aggregation occurred. For all BSA concentrations, pH was slightly decreased when the molecular weight of CG was higher. Although, the molar ratio of glucosamine (CG 35 and CG 45) and glutamic acid was 1:1 mole, but the amount of glutamic acid used for dissolving CG 45 was higher than CG 35. So, it may cause the lower pH when the higher molecular weight of CG was used. The increase in BSA concentration gave the slightly increased pH. All molecular weights of chitosan showed the positive charge indicated the unoccupied amine group. The zeta potential was in the range of 24-38 mV. The addition of BSA in both molecular weights of CG caused the lower zeta potential due to the negative charge from BSA, indicating the interaction between CG and BSA. The higher molecular weight did not significantly differ in zeta potential, due to slight difference in the molecular weight and the addition of BSA at CG 45 from 0.5 to 1.0 mg/mL did not affect the zeta potential.
Figure 42  Effect of different molecular weight of CG and concentrations of BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (HY 30 0.200 %w/v, CG 0.100 %w/v)
The particle sizes were in the range of 117-140 nm, and were not different in distinct of molecular weight. The addition of BSA caused the decrease in the particle size of CG35, but for CG45, the particle size tended to increase at the same amount of BSA. Hence, the molecular weight of chitosan had an influence on the formation and property of nanoparticle. At CG 0.100 and HY 30 0.200 %w/v, the BSA could be loaded at the concentration of 0-0.5 mg/mL for CG 35 while for CG 45 the wider range of BSA (0-1.0 mg/mL) could be incorporated. The higher concentration of BSA led to the aggregation. Therefore, the optimum concentrations of CG, HY and BSA including molecular weight of CG contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.

4.4.1.2 Effect of molecular weight of CG on % encapsulation efficiency (% EE) and loading efficiency (% LE) of BSA-loaded CG-HY_HY NP

The % EE and % LE are shown in Figure 43. The increase in MW of CG at the fixed concentration of BSA showed the significant decrease in % EE and % LE ($p<0.05$). This could be the result of the increase in the viscosity which was correlated with the fact that the increase in the molecular weight made it difficult for the encapsulation to take place by averting the BSA molecular movement around the chitosan molecule chain [21, 226]. In addition, the low MW of chitosan solution containing the shorter fragments made its free amino group easier to protonate and then led to greater encapsulation of BSA through ionic interactions [234]. However, the opposite result was found for the work of Xu and Du [25]. The higher %EE of high MW was due to the gelation effect inducing by TPP and the ability of entrapment of BSA by the longer chain. The higher amount of BSA of CG45 had a tendency to increase in the EE and LE which was similar to the work of Gan and Wang [21] whereas the opposite effect was reported by other studies [25, 221]. The higher EE and LE with the increase in BSA was also in accordance with the reduction in the zeta potential confirming that there was an interaction between the positive charge of CG 45 and the negative charge of BSA. However, the addition of BSA at CG45 from 0.5 to 1.0 mg/mL did not affect the zeta potential.
Figure 43 Effect of different molecular weight of CG and concentrations of BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (HY 30 0.200 %w/v, CG 0.100 %w/v)

4.4.2.1 Effect of different hydrolysis times of shellac on pH, zeta potential and particle size of BSA-loaded CG-HY_HY NP

Table 19 shows the effect of different hydrolysis time of shellac on the formation of BSA loaded nanoparticles. The nanoparticle could be formed for all hydrolysis times of shellac at 0 and 0.5 mg/ml BSA and the higher amount of BSA
leading to aggregation occurred. The aggregation was reported with the increase in BSA concentration in the range between 1.0-2.0 mg/mL for all hydrolysis time of shellac. The aggregation was the result of the reduction in repulsive force then the particles became closer and aggregation occurred.

Table 19 Effect of different hydrolysis time of shellac and concentrations of BSA on the formation of nanoparticles (HY 0.200 %w/v, CG 35 0.100 %w/v)

<table>
<thead>
<tr>
<th>BSA (mg/mL)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>0.5</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>1.0</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
</tr>
<tr>
<td>1.5</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
</tr>
<tr>
<td>2.0</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
</tr>
</tbody>
</table>

NP = Nanoparticle formation  
Ag = Aggregation state

The effect of different hydrolysis time of shellac on the pH, zeta potential and particle size are shown in Figure 44. The increase in the hydrolysis time of shellac, the lower pH was obtained. The result was correlated with the higher AV with the increase in hydrolysis time [30, 215]. The addition of BSA did not change in pH for all hydrolyzed shellacs. The zeta potential of all nanoparticles displayed a positive charge and was in the range between 21 – 41 mV. The increase in hydrolysis time of shellac did not result in the reduction of zeta potential but the opposite result was noted. This result suggested that the increase in the zeta potential with the increase in hydrolysis time of shellac was attributed to the increase in the unoccupied amine group of CG due to the increased competitiveness of deprotonated carboxylic groups between HY and BSA causing the difficulty in interaction with CG [227]. The particle size of the colloid was in the 105-250 nanometer size range as shown in Figure 44. The higher hydrolysis time, the lower size was obtained due to the highest amount of negative charge giving the tighter bonding and smaller size obtained [30].
Figure 44 Effect of different hydrolysis time of shellac and concentrations of BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (mean±S.D., n=3)( HY 0.200 %w/v, CG 35 0.100 %w/v)
The addition of BSA caused the increase in particle size except the native shellac showed the significant decreased size \((p<0.05)\). At CG 35 0.100 and HY 0.200 %w/v, the nanoparticle could be formed at BSA 0-0.5 mg/mL of of all hydrolysis times of HY. Therefore, the optimum concentrations of CG, HY and BSA including hydrolysis time of HY contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.

Figure 45  Effect of different of hydrolysis time of shellac and concentrations of BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (HY 0.200 %w/v, CG 35 0.100 %w/v)
4.4.2.2 Effect of different hydrolysis times of shellac on % encapsulation efficiency (% EE) and loading efficiency (% LE) of BSA-loaded CG-HY_HY NP

The effect of different hydrolysis time of shellac on the % EE and % LE of nanoparticle is shown in Figure 45. The increase in hydrolysis time of shellac at the fixed concentration of BSA showed significant decrease in % EE and % LE ($p<0.05$). The lower of % EE and % LE with the increase in hydrolysis time of shellac was also in accordance with the increase in the zeta potential (between 23-31 mV) indicating the unoccupied amine of chitosan and hence the lower %EE and %LE.[227]. The lower %EE and %LE were the result of the increase in carboxylic group of HY and the smaller structure with the increased hydrolysis causing the higher competitiveness between HY and BSA [227]. Therefore, the longer hydrolysis time attributed to the higher interaction between HY and CG and lower %EE and %LE.

Table 20 Effect of different concentrations of CG 35 kDa and BSA on the formation of nanoparticles and aggregation state

<table>
<thead>
<tr>
<th>BSA (mg/mL)</th>
<th>CG 35 (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.075</td>
</tr>
<tr>
<td>0</td>
<td>NP</td>
</tr>
<tr>
<td>0.5</td>
<td>Ag</td>
</tr>
<tr>
<td>1.0</td>
<td>Ag</td>
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<tr>
<td>1.5</td>
<td>Ag</td>
</tr>
<tr>
<td>2.0</td>
<td>Ag</td>
</tr>
</tbody>
</table>

NP = Nanoparticle formation  
Ag = Aggregation state

4.4.3.1 Effect of different concentrations of CG 35 on pH, zeta potential and particle size of BSA-loaded chitosan-hydrolyzed shellac nanoparticles

Table 20 shows the effect of different concentrations of CG 35 kDa on the formation of BSA loaded nanoparticles. Two physical states were reported ie, nanoparticle and aggregation states. At higher concentration of CG 35 nanoparticles could be formed for all concentrations of BSA while at the lower concentrations of CG 35 such as 0.075 and 0.100 %w/v, the nanoparticles could be formed at only the
lower concentration of BSA. However, solution state was not reported in all concentrations of CG and BSA. The aggregation was reported with the increase in BSA concentration in the range between 0.5-2.0 mg/mL and 1.0-2.0 mg/mL for the concentration of CG 35 at 0.075 and 0.100 %w/v, respectively. The aggregation was the result of the reduction in repulsive force when higher amount of negatively charged BSA was incorporated into the lower concentration of positively charged CG 35. Hence, the particles became closer and aggregation occurred [18, 20, 227]. The increase in concentration of CG 35 at the fixed concentration of HY 30 and BSA, the lower pH was obtained for all concentrations of BSA. The increased BSA at all concentrations of CG 35 gave the slightly increased pH. The zeta potential was increased with the increase in concentration of CG 35 due to the increase in positive charge from the amine groups of chitosan [18, 227] (Figure 46). The incorporation of BSA gave the lower zeta potential. The higher BSA, the lower zeta potential was reported. The result proved that the BSA showed a negative charge as the pH being studied was higher than the pI value (pI=4.7, the pH under this condition was approximately 5.5) which was in agreement with the result in preliminary section [17, 97, 235]. The highest reduction in zeta potential was reported for CG 35 at 0.125% w/v and 2 mg/ml BSA. The particle size of different concentrations of CG 35 were around 99-267 nm. In general, the increase in BSA concentration gave the lower size for all concentrations of CG except at 0.125% w/v CG and 2 mg/ml BSA. The tremendously increased size was reported as a result of the highest reduction in zeta potential. At HY 30 0.200 %w/v and CG 35 at 0.125-0.175 %w/v, the nanoparticle could be formed at all concentrations of BSA while at the 0.075 and 0.100 %w/v of CG 35, the nanoparticles could be formed only at BSA 0-0.5 mg/mL. Therefore, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.
Figure 46  Effect of different concentrations of CG 35 and BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (HY 30 0.200 %w/v)
4.4.3.2 Effect of different concentrations of CG 35 on % encapsulation efficiency (% EE) and loading efficiency (% LE) of BSA-loaded CG-HY_HY NP

The % EE and % LE were in the range of 11-76 % and 3-45 % as shown in Figure 47, respectively. The increase in the concentrations of CG 35 caused the significant ($p<0.05$) decrease in the % EE and % LE, which was in agreement with the preliminary study and with other report [21]. The decrease in % EE and % LE was consistent with the increase in the zeta potential indicating the higher unoccupied amine of CG with the increase in CG 35. The higher amount of BSA had a tendency to increase in the % EE and % LE which was similar to the work of Gan and Wang [21] whereas the opposite effect was reported by other studies [25, 221]. The higher in % EE and % LE with the increase in BSA was also in accordance with the reduction in the zeta potential confirming that there was an interaction between the positive charge of CG and the negative charge of BSA. In this study, the highest % EE and % LE (76 and 45 %w/v, respectively) was obtained for CG 35 at the concentration of 0.125% w/v and was in agreement with the lowest zeta potential indicating the highest interaction of BSA and amine group of CG. So, the suitable concentration of CG 35, HY 30 and BSA to form nanoparticle were 0.125 %w/v, 0.200 %w/v and 2 mg/mL, respectively since it can give the highest %EE and %LE. However, after 1 - 2 h of storage, the nanoparticle had a tendency to aggregate. Hence, the second high of %EE and %LE was chosen for further study in the next section ie, 0.125% w/v CG 35, 0.2%w/v HY30 and 1.5 mg/mL BSA. It could be concluded that the studied concentration of CG could be used to form nanoparticle. Unlike the HY0, the higher range of concentration of CG and the higher %EE and % LE was obtained for HY 30.
Figure 47 Effect of different concentrations of CG 35 and BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (HY 30 0.200 %w/v)
4.4.4.1 Effect of different concentrations of HY 30 on pH, zeta potential and particle size of BSA-loaded CG-HY_HY NP

Table 21 shows the effect of different concentrations of HY 30 on the formation of nanoparticles. Three physical states were reported ie, solution, nanoparticle and aggregation states. At the low concentration of HY 30 such as 0.150 and 0.175 %w/v at the BSA 0 mg/mL the solution state was displayed. The solution state indicating that each molecule of CG, HY and BSA was apart. This could be due to the greater repulsive force than the attractive force at the lower BSA and HY 30 concentration. This caused the higher unoccupied amine group of chitosan and the higher zeta potential and hence the solution state was formed. The result showed that in some certain concentrations of the charged particles, the nanoparticle could be formed. At higher concentration of HY 30 nanoparticles could be formed for all concentrations of BSA. The formation of nanoparticles arises from the balance between the repulsive and attractive forces. The aggregation was reported with the increase in BSA concentration in the range between 1.0-2.0 mg/mL for the concentration of HY 30 at 0.250 %w/v, respectively. The aggregation was the result of the reduction in repulsive force when higher amount of negatively charged BSA and HY 30. Hence, the particles became closer and aggregation occurred. The result was in agreement with previous study of native shellac [227].

Table 21 Effect of different concentrations of HY 30 and BSA on the formation of nanoparticles, solution and aggregation state (CG 35 0.125 %w/v)

<table>
<thead>
<tr>
<th>BSA (mg/mL)</th>
<th>HY 30 (% w/v)</th>
<th>0.150</th>
<th>0.175</th>
<th>0.200</th>
<th>0.225</th>
<th>0.250</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NP</td>
<td>NP</td>
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</tr>
<tr>
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<td>NP</td>
<td>NP</td>
<td>NP</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>1.5</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>Ag</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>Ag</td>
<td></td>
</tr>
</tbody>
</table>

NP = Nanoparticle formation
Ag = Aggregation state
Sol = Solution state
Figure 48  Effect of different concentrations of HY 30 and BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (CG 35 0.125 %w/v)
Figure 48 shows the effect of concentration of HY 30 on pH, zeta potential and particle size of BSA-loaded CG-HY_HY NP. The increase in concentration of HY 30 at the fixed concentration of BSA, the lower pH value was obtained. This might be due to the higher amount of HY 30 attributing to the increase in AV of HY 30. The zeta potential of various concentrations of HY 30 was not significantly different at the fixed concentration of BSA. Similar to other factors, the increase in BSA gave the lower zeta potential. The particle size of different concentration of HY 30 were around 96-273 nm. The increase in BSA concentrations from 0-1.5 mg/ml did not cause the change in size for all concentrations of HY30. However, at 2.0 mg/ml BSA the significant increase in size was obtained (p<0.05). The particale size increased from 100 nm to around 250 nm for all concentrations of HY 30. The increase in particle size correlated with the reduction in zeta potential (lower than 20 mV) such that the particle became closer and hence the larger size obtained. [20]. At CG 35 0.125 %w/v and HY 30 at 0.200-0.225 %w/v, the nanoparticle could be formed all concentrations of BSA while at the 0.250 %w/v of HY 30, the nanoparticles could be formed only at BSA 0-0.5 mg/mL. At HY 30 0.150-0.175 %w/v, the nanoparticle could be formed all concentrations of BSA excepting without BSA. Therefore, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.

4.4.4.2. Effect of different concentrations of HY 30 on % encapsulation efficiency (% EE) and loading efficiency (% LE) of BSA-loaded CG-HY_HY NP

The % EE and % LE were in the range of 15-76% and 5-46% as shown in Figure 49, respectively. The result was in the same manner as the preliminary study that the increase in concentrations of native shellac did not correlate to % EE and % LE. At BSA 0.5-1.0 mg/mL, the increase in concentration of HY 30 from 0.150-0.200 %w/v caused the slight increase in % EE and % LE. In addition, %EE and %LE at 0.225 %w/v of HY 30 were not significantly different with 0.200 %w/v at the same concentration of BSA. At BSA 1.5-2.0 mg/mL, the % EE and % LE of concentration of HY 30 at 0.200 %w/v was higher than other concentrations.
Figure 49  Effect of different concentrations of HY 30 and BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (CG 35 0.125 %w/v)
This appearance associated with the using of native shellac for nanoparticle formation that the concentration of HY 0 at 0.050 %w/v gave the highest % EE and % LE than other concentrations. In addition, at BSA 2.0 mg/mL, the % EE and LE were higher and was comply with the higher reduction in zeta potential indicating the higher interaction between BSA and CG. [227, 235]. BSA concentration in the range between 0.5-2.0 mg/mL and the concentration of HY 30 between 0.150-0.250 %w/v could be prepared the nanoparticle which the concentrations of HY 30 were wider range than using HY 0 as previously described to form the nanoparticle.

4.4.5.1 Effect of pH adjustment and different concentrations of BSA on pH, zeta potential and particle size of BSA-loaded CG-HY_HY NP

pH value plays an important role for nanoparticle formation. Table 22 shows the effect of pH adjustment of different concentrations BSA on the formation of nanoparticles. There were 2 physical states formed, ie. nanoparticle and aggregation. The blank nanoparticle could be formed at all initial pHs. At pH 5.5, the aggregation occurred with the addition of BSA. At initial pH 5.25, the nanoparticles could be formed in all concentrations of BSA except at BSA 1.5mg/mL, the aggregation occurred. The concentration of HY 30, CG 35 and BSA at 0.200 %w/v, 0.125 %w/v and 0-1.5 mg/mL, respectively were chosen to form nanoparticle in this study because they can keep in nanoparticle size range and gave the highest in % EE and % LE. At BSA 2.0 mg/mL was not chosen in this section since it was instability after 1-2 hrs of flesh preparation and the aggregation was occurred with the higher pH adjustment. As shown in Figure 50, the increased pH adjustment, the higher pH was obtained for all concentrations of BSA. The addition of all concentrations of BSA with the increase in pH adjustment did not significantly affect the pH value compared with blank nanoparticle. The higher pH adjustment caused the reduction in zeta potential for all concentrations of BSA. The decrease in zeta potential after increasing of initial pH may be due to the reduction of positive charge from the amine groups of CG (pKa of chitosan ~ 6.5) and higher negative charge from the anionic groups of BSA [227, 235] which correlated with Gan et al. that showed a continual decrease in the positive zeta potential after increasing in initial pH. It can be described by the degree of protonisation at surface of the particles were reduced and then decreasing
electrostatic repulsion between the particles thereby increasing the probability of particle aggregation [20]. The pH of higher pKa of CG caused the lower ionization and lower positive charge obtained [17]. The particles size were around 99-225 nm. At BSA 1.0 and 1.5 mg/mL of pH adjustment at 4.75 and 5, the lower particle size than other pH were obtained. It was correlated with the result of zeta potential that gave the lower than others. Hence, the pH adjustment at 4.75 and 5 were suitable to form nanoparticle because of smaller particle size than other pH [20]. In addition, they were higher in %EE and %LE than others that it will be discussed in next section. So, the optimum of pH adjustment of CG had an importance for formation of nanoparticle. At CG 35 0.125 %w/v, HY 30 at 0.200 %w/v and initial pH at 4.0-5.0, the nanoparticle could be formed all concentrations of BSA while at the initial pH of 5.25, the nanoparticles could be formed only at BSA 0-1.0 mg/mL. At initial pH 5.5, the nanoparticle could be formed only without BSA. Therefore, the optimum concentrations of CG, HY and BSA including pH adjustment contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.

Table 22 Effect of pH adjustment of different concentrations BSA on the formation of nanoparticles and aggregation state (CG 35 0.125 %w/v, HY 30 0.200 %w/v)

<table>
<thead>
<tr>
<th>BSA (mg/mL)</th>
<th>Initial pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.00</td>
</tr>
<tr>
<td>0</td>
<td>NP</td>
</tr>
<tr>
<td>0.5</td>
<td>NP</td>
</tr>
<tr>
<td>1.0</td>
<td>NP</td>
</tr>
<tr>
<td>1.5</td>
<td>NP</td>
</tr>
</tbody>
</table>

NP = Nanoparticle formation
Ag = Aggregation state
Figure 50  Effect of pH adjustment of different concentrations BSA on (A) pH, (B) zeta potential and (C) particle size of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (CG 35 0.125 %w/v, HY 30 0.200 %w/v)
4.4.5.2. Effect of pH adjustment and different concentrations of BSA on % encapsulation efficiency (% EE) and loading efficiency (% LE) of BSA-loaded CG-HY_HY NP

The % EE and % LE were around 7-79% and 2.5-33%, respectively as shown in Figure 51. The %EE and %LE associated with the zeta potential that the higher in EE and LE with the increase in pH was also in accordance with the reduction in the zeta potential confirming that there was an interaction between the positive charge of CG and the negative charge of BSA [227]. The highest %EE and %LE was found at pH 5.25, 1.0 mg/mL BSA and the nano size range was obtained. The result of %EE and %LE was in agreement with the lowest zeta potential. [227, 235]. However, at this pH, it had a tendency to aggregate after 1 or 2h of complete preparation. So, the initial pH at 4.75 and 5 could give higher in %EE and %LE than other ones as the same concentration of BSA. This result confirmed the optimum of initial pH that used for preparing the nanoparticle. If the initial pH was over 5, the aggregation may be occurred, and in contradiction, if the initial pH was lower than 4.75, the low %EE and % LE will be obtained.
Figure 51 Effect of pH adjustment of different concentrations BSA on (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) of BSA-loaded CG-HY_HY NP (mean±S.D., n=3) (CG 35 0.125 %w/v, HY 30 0.200 %w/v)
4.5 Enteric-coated capsules filled with freeze-dried BSA-loaded CG-HY_HY NP

4.5.1 In vitro of BSA release

From preliminary study, BSA could be released from the nanoparticles in phosphate buffer pH 7.4 that was simulated to the body fluid. However, BSA-loaded CG-HY_HY NP could not protect BSA from the SGF pH 2.0 and the immediate release of BSA from the matrix at around 70-87 % was displayed in Figure 52. This problem was solved by using the enteric coated capsule containing the freeze-dried nanoparticles for the protection of the hazard environment in stomach and intestine. The effect of various factors on the release such as, MW, hydrolysis time, concentration of CG 35, HY 30 and BSA was investigated as shown in Figures 53-57. The concentration of CG, HY and BSA at 0.125, 0.200 and 1.5 mg/mL were chosen because it can maintain in nanometer size range and gave the higher in %EE and % LE than the others. Although, the BSA 2.0 mg/mL gave the highest in %EE and %LE (around 80 %) but it had a tendency to aggregate after 1 or 2 h of fresh preparation. The effect of molecular weights of CG is shown in Figure 53. There was no release for the first 2 hours of study in SGF pH 2.0. This proved that the Eudragit L enteric coated capsule could be used to protect the BSA release from the freeze dried nanoparticles. In SIF pH 6.8, the enteric coated capsule was gradually disintegrated after 30 minutes and slow release was reported. The completely constant release was found in SIF pH 7.4 after 8 hours of study. The % cumulative release was between 67.9-75.4 %. The release of BSA for CG35 was lower than the CG 45 (p<0.05), and the result was opposite to the work of Xu & Du [25]. However, the influence of molecular weight of polymer on the mechanism of drug or protein release from nanoparticles has not been explained clearly. Figure 54 shows the effect of hydrolysis times of shellac on BSA release. The % cumulative release was between 67.8-70.9 %. Varying hydrolysis times of shellac did not cause the release of BSA from the matrix significantly different (p>0.05). Figure 55 shows the effect of concentrations of CG 35 on BSA release. The % cumulative release of all concentrations of CG 35 was between 67.9-71.6 %. The lowest release was found for 0.125 %w/v, as a result of the larger size. The larger size attributed to the larger diffusion path length for the BSA and the lower contact surface area of the larger particles with the dissolution medium [13, 227]. The effect of concentrations of HY
30 on BSA release is shown in Figure 56. The only three concentrations of HY 30 (0.150, 0.175 and 0.200 %w/v) were chosen in the release study and at 0.225 %w/v of HY 30 was not chosen because it had tendency to aggregate after preparing for 2 h. So, the concentrations of HY 30 (0.150, 0.175 and 0.200 %w/v) were representative effect of different concentration of HY 30 in release study. The cumulative releases of BSA from the various concentrations of HY 30 were around 67.9-71.6 %, the release of these 3 concentrations was not different significantly ($p > 0.05$). The effect of different concentrations of BSA is shown in Figure 57. The cumulative amounts of BSA release from various concentrations of BSA were around 67.9-80.2 %. The release of BSA at 2.0 mg/mL was lower than the others ($p < 0.05$), due to the larger size ($\sim 330$ nm) [13, 227]. This result was correlated with the highest release of BSA at 1.5 mg/mL that it had a smaller size than the others. Therefore, it could be concluded that the release of BSA from the particulate systems was dependent on the size and the Eudragit L enteric coated capsule could be used to protect the BSA release from the freeze dried nanoparticles in SGF pH 2.0. In SIF pH 6.8 and 7.4, the % cumulative release of all conditions was between 60.0-80.0 % and only varying BSA concentration had a significant effect on the % cumulative release. The important factors that had an influence on the % cumulative release of BSA were surface area and particle size. Hence, the release of nanoparticle could be modified by the concentrations of BSA and particle size.
Figure 52  The release profile of BSA-loaded CG-HY_HY NP in SGF pH 2.0, 37 °C, (mean ± SD, n = 3). (CG 35 0.125 %w/v, HY 30 0.200 %w/v)

Figure 53  The release profile of BSA-loaded CG-HY_HY NP at different molecular weights of CG in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (mean ± SD, n = 3). (CG 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL)
Figure 54  The release profile of BSA-loaded CG-HY_HY NP at different hydrolysis time of shellac in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (mean ± SD, n = 3). (CG 35 0.125 %w/v, HY 0.200 %w/v and BSA 1.5 mg/mL)

Figure 55  The release profile of BSA-loaded CG-HY_HY NP at different concentrations of CG 35 in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (mean ± SD, n = 3). (HY 30 0.200 %w/v, BSA 1.5 mg/mL)
Figure 56 The release profile of BSA-loaded CG-HY_HY NP at different concentrations of HY 30 in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (mean ± SD, n = 3). (CG 0.125 %w/v, BSA 1.5 mg/mL)

Figure 57 The release profile of BSA-loaded CG-HY_HY NP at different concentrations of BSA in SGF pH 2.0, SIF pH 6.8 and SIF pH 7.4, 37 °C, (mean ± SD, n = 3). (CG 0.125 %w/v, HY 30 0.200 %w/v)
4.6 Stability of nanoparticles

It has been reported that nanoparticles were not stable upon storage, the aggregation was noted [235]. The stability of NP in colloidal form was investigated at 4°C for 1 month. The comparison was made between those with and without cryoprotectant as shown in figure 58-59.

4.6.1 Stability of BSA-loaded CG-HY_HY NP kept in colloidal form at 4 °C

4.6.1.1 pH, zeta potential and particle size

The stability of nanoparticle in colloidal was carried out at 4 °C for 1 month. The change in pH, Zeta potential, particle size and %EE and LE of the formulation with and without Glu were investigated as shown in Figures 58-59.

Figure 58 shows the stability of BSA-loaded CG-HY_HY NP in colloidal form in terms of pH, Zeta potential and particle size. The pH of all formulations after 1 month storage had slightly increased. The storage time resulted in the significant decrease in the zeta potential of all formulations (with and without Glu) after 1 month of storage (p<0.05). The aggregation of particle was occurred when amount of BSA of all formulations had more than 1.0 mg/mL and were kept for 1 month. This result indicated that the reduction in the zeta potential until it reached a certain point causing the minimum of repulsive force between the nanoparticles leading to the larger or aggregation of particles [18, 20]. In this study, the zeta potential was reduced to around 20 mV. This caused the decrease in the electrostatic repulsion force between the particles leading to the aggregation of particles. The aggregation was found for the formulation with the loading of BSA more than 0.5 mg/ml. Hence, it could be concluded that Glu as a cryoprotectant could not stabilize the nanoparticle which was prepared in colloidal form for 1 month of storage time.
Figure 58  Effect of stability of BSA-loaded CG-HY_HY NP kept in colloidal form at 4 °C on (A) pH, (B) zeta potential and (C) particle size (mean±S.D., n=3) (CG 35 0.125 %w/v, HY 30 0.200 %w/v, and Glu 7.5 %w/v)
Figure 59 Effect of Glu on stability of BSA-loaded CG-HY_HY NP in colloidal form at 4 °C: (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (mean±S.D., n=3) (CG 35 0.125 %w/v, HY 30 0.200 %w/v and Glu 7.5 %w/v)
4.6.1.2. % encapsulation efficiency (% EE) and loading efficiency (% LE)

Figure 60 shows the effect of Glu on the %EE and %LE of the nanoparticle. All formulations at the beginning of storage time, the encapsulation and loading efficiency were increased with the increase in the concentration of BSA. The effect of storage time on the %EE and LE did not behave differently. At 0.5 mg/ml BSA those without Glu had the same as %EE and LE was found for those with Glu. Beyond 0.5 mg/ml, their efficiency of both with and without Glu at 1 month could not be detected due to the aggregation. Therefore, the Glu could not be used to protect the nanoparticle in the colloidal form.

Figure 60  Physical photograph of the nanoparticle; before freeze drying (A), and after freeze drying (B) (using Glu as a cryoprotectant)

4.6.2 Various factors on the stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C

4.6.2.1 Effect of different concentrations of CG 35 on pH, zeta potential and particle size

The dried powder form has been suggested for prolonging shelf life. Therefore, in this study, nanoparticle was prepared into dried form by using freeze drying process. Glucose (Glu) was used as a cryoprotectant prior to freeze drying process. The photograph of the nanoparticle was taken before and after freeze drying process with the addition of Glu as shown in Figure 60. The large particle size and aggregation was found in the formulation without Glu as a cryoprotectant after it
Figure 61  Effect of different concentrations of CG 35 on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C, (A) pH, (B) zeta potential and (C) particle size (mean±S.D., n=3) (HY 30 0.200 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)
was freeze-drying and redispersed with distilled water (data not shown). So, Glu was used to protect the nanoparticle from the freeze drying process. The nanoparticles with the aid of Glu were then prepared into powder form at 3 concentrations of CG 35 (0.125, 0.150 and 0.175 %w/v) and the stability of dried powder was carried out for 3 months as shown in Figure 61. These concentrations of CG 35 were chosen because they can be formed the nanoparticle in all concentrations of BSA differing from the concentrations of CG 35 at 0.100 and 0.075 %w/v that it can be formed the nanoparticle with only BSA 0.5 mg/mL and without BSA. In addition, at BSA 2.0 mg/mL was not chosen due to the instability of the nanoparticle after freshly prepared for 1-2 h. The higher concentration of CG 35 showed the lower pH. The 3-month storage time did not change the pH of nanoparticle for all concentrations of CG 35 as shown in Figure 61A. Figure 61B shows the effect of storage time on the zeta potential. The higher concentration of CG 35, the higher zeta potential was reported. The storage time caused the slight reduction in zeta potential for all concentrations. The nanoparticle sizes of all concentrations were in the range of 100-120 nm. The storage time did not result in the change in particle size which was in accordance with the slight change in zeta potential [18, 235]. The opposite result was reported for the storage in the colloidal form for 3 months which found the lower in zeta potential and leading to the aggregation. Therefore, it could be concluded that the cryoprotectant could be used to stabilize the nanoparticle in dry form for 3 months of storage and the concentration of CG 35 had no effect on the stability of nanoparticles. Additionally, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.

4.6.2.2 Effect of different concentrations of CG 35 on % encapsulation efficiency (% EE) and loading efficiency (% LE)

Although, the various concentrations of CG 35 had a slightly effect on the physiochemical characteristic of the nanoparticles after 3 months of storage time, it had an effect on the % encapsulation and loading efficiency as shown in Figure 62. The lowest concentration of CG displayed the highest %EE and %LE at initial. After 3 months of storage, the %EE and %LE decreased significantly (p<0.05) for all concentrations. It may be due to the high hygroscopic property of Glu [199]
and the absorbed water may dissolve some BSA from the nanoparticle resulting in the reduction in %EE and %LE.

Figure 62: Effect of different concentrations of CG 35 on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C. (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (mean±S.D., n=3) (HY 30 0.200 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)
4.6.3.1 Effect of different concentrations of HY 30 on pH, zeta potential and particle size

The effect of different concentrations of HY 30 on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C after redispersed with distilled water is shown in Figure 63. The pH of initial time and storage time for 3 months of various concentrations of HY 30 did not significantly differ. The zeta potential at initial time was decreased at higher concentration of HY 30 due to the higher amount of negative charge of HY 30 [20]. After 3 months of storage, the zeta potential changed differently. For the lowest concentration, the zeta potential decreased upon storage while the other concentrations were stable. The zeta potential for the lowest concentration was reduced to 15 mV after 3 months of storage. Figure 63C shows the result of effect of storage time, all the particle sizes were in the range around 103-120 nm. The time of storage did not affect the growth of particle size for all concentrations. Although the zeta potential of the lowest concentration of HY 30 was reduced to 15 mV, which was lower than in colloidal form, the particle was still remain in nanometer range. The concentration of HY 30 did not affect the stability of freeze dried nanoparticle after storage for 3 months. So, this could be proved that the nanoparticle in dried powder form with the aid of Glu could prevent the particle from aggregation. Additionally, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.
Figure 63  Effect of different concentrations of HY 30 on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C, (A) pH, (B) zeta potential and (C) particle size (mean±S.D., n=3) ( CG 35 0.125 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)
4.6.3.2 Effect of different concentrations of HY 30 on % encapsulation efficiency (% EE) and loading efficiency (% LE)

Figure 64 shows the effect of concentrations of HY 30 on stability, encapsulation and loading efficiency of the nanoparticles. The %EE and %LE were around 25-48% and 13-24%, respectively. The %EE and %LE of initial time of HY 30 at concentration of 0.15 %w/v and 0.175 %w/v were lower than those of 0.2 %w/v. The storage time did not affect the %EE and %LE for the concentrations of 0.150 and 0.175 %w/v, whereas for the concentration of 0.2 %w/v had a tendency to decrease after 3 months of storage. It may be due to the high hygroscopic property of Glu and the absorbed water may dissolve some BSA from the nanoparticle resulting in the reduction of %EE and %LE. However, at the concentrations of 0.150 and 0.175 %w/v did not affect by this solubilizing effect of glu upon storage. In addition, it may be due to the increase in adsorption of BSA on the surface of nanoparticle after storage for a long time resulting in the immediate release of BSA from the nanoparticle.
Figure 64 Effect of different concentrations of HY 30 on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (mean±S.D., n=3) (CG 35 0.125 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)
4.6.4.1 Effect of different hydrolysis time on pH, zeta potential and particle size

Effect of different hydrolysis time of shellac on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C is shown in Figure 65. The pH of nanoparticle at beginning of storage time were in the range of 5.42-5.48 of each hydrolysis time of shellac. At 3 months, pH did not change from the initial when the hydrolysis time was longer for all hydrolysis time. At initial, the increased hydrolysis time (from 0-30 min) had no effect on zeta potential but for 45-min hydrolyzed shellac was higher. The result was attributed to the increase in the unoccupied amine group of CG due to the higher negative charge of HY 45 causing the higher competitiveness of deprotonated carboxylic groups between HY 45 and BSA more than other hydrolysis times [30, 215, 227]. The effect of hydrolysis time did not cause the change in the particle size and the storage time did not cause the change in particle size for all hydrolyzed shellacs. This could prove that the preparation of all hydrolyzed shellacs in dried powder form with the addition of Glu could be used to improve the stability of all hydrolyzed shellacs. Additionally, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.
Figure 65  Effect of different hydrolysis time of shellac on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C (A) pH, (B) zeta potential and (C) particle size (mean±S.D., n=3) ( CG 35 0.125 %w/v, HY 0.200 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)
4.6.4.2 Effect of different hydrolysis time on % encapsulation efficiency (% EE) and loading efficiency (% LE)

Figure 66 shows the effect of different hydrolysis time on stability, encapsulation and loading efficiency of the nanoparticle. The %EE and %LE were in the range 19-48 and 10-24, respectively. The %EE and %LE of initial time was increased with the increased hydrolysis time and showed the maximum at HY 30. For HY 45, the lower %EE and %LE were the result of the increase in carboxylic group of HY causing the higher competitiveness between HY and BSA [227]. So, the hydrolyzed shellac could interact with CG easier than BSA which caused the lower %EE and %LE. The 3-month storage caused the decrease in %EE and %LE for all hydrolyzed shellacs and the highest %EE and %LE was noted for HY 30 and was correlated with the lowest zeta potential. It may be due to the high hygroscopic property of Glu and the absorbed water may dissolve some BSA from the nanoparticle resulting in the reduction in %EE and %LE.
Figure 66 Effect of different hydrolysis time of shellac on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (mean±S.D., n=3) (CG 35 0.125 %w/v, BSA 1.5 mg/mL and Glu 7.5 %w/v)
4.6.5.1 Effect of different concentrations of BSA on pH, zeta potential and particle size

Effect of different concentrations of BSA on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C is shown in Figure 67. The pH of nanoparticles of all concentrations of BSA had a slight increase when it was kept for 3 months. The zeta potential of all concentrations of BSA almost did not change after 3 months of storage time. The particles size were in the range of 99-220 nm at the concentrations of BSA between 0-1.5 mg/mL, but the concentration of 2.0 mg/ml displayed the largest size around 330 nm at the beginning of storage time. After 3 months, BSA concentrations in the range of 0-1.5 mg/mL, the particles could be maintained in the nano size range, but the aggregation state was exhibited at the concentration of BSA 2.0 mg/mL. The unstable particle might be a result of insufficient cryoprotectant [236] and the lowest zeta potential for 2.0 mg/ml BSA giving the lowest repulsive force [227, 235]. The reduction in the zeta potential lower than 20 mV of the nanoparticles caused the nanoparticles to come closer until it was aggregated. Hence, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles. In next section, the effect of concentration of cryoprotectant was performed.
Figure 67  Effect of different concentrations of BSA on stability of BSA-loaded CG-HY HY NP at kept in dried form at 4 °C (A) pH, (B) zeta potential and (C) particle size (mean±S.D., n=3) ( CG 35 0.125 %w/v, HY 30 0.200 %w/v and Glu 7.5 %w/v)
4.6.5.2 Effect of different concentrations of BSA on % encapsulation efficiency (% EE) and loading efficiency (% LE)

As shown in Figure 68 is the effect of different concentrations of BSA on stability, % encapsulation and loading efficiency of the nanoparticle. The %EE and %LE were in the range of 13-72% and 5-43%, respectively. An increase in %EE and %LE were found with the increase in concentration of BSA at the beginning of storage time which was similar to the work of Gan and Wang [21, 227]. The effect of storage time had an influence on the %EE and %LE depending on the concentration of BSA. The longer time tended to increase the %EE and LE but the opposite result was for 1.0 mg/ml BSA. In addition, for BSA 2.0 mg/ could not be investigated due to the aggregation. Hence, the optimum concentration of BSA to form nanoparticle was 1.5 mg/mL since it gave the smaller size and the higher in %EE and %LE than another one.
Figure 68  Effect of different concentrations of BSA on stability of BSA-loaded CG-HY_HY NP at kept in dried form at 4 °C) encapsulation efficiency (EE) and (B) loading efficiency (LE) (mean±S.D., n=3) (CG 35 0.125, HY 30 0.200 %w/v and Glu 7.5 %w/v)
4.6.6.1 Effect of different concentrations of Glu and trehalose (Tre) on pH, zeta potential and particle size

Effect of different concentrations of Glu and Tre on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C is shown in Figure 69. The cryoprotective effect was attributed to the ability of the sugar additive to form a glassy amorphous matrix around the particles, preventing the particles from sticking together during the removal of water[237]. In general, the level of stabilization provided by sugars is depended on their concentrations [199, 236-238]. The pH of initial and 3 months of storage time of all concentrations and types of cryoprotectant were 5.40-5.50. The increase in concentration of the same cryoprotectant had a slight effect on the zeta potential since it was a non-ionized material [199]. The pH and the zeta potential of different concentrations of Glu and Tre did not differ from the beginning after storage for 3 months. The particles size were in the range of 105-140 nm, but it was only 5 % w/v of Tre displaying larger size of particle than the other concentrations, and it had a trend to aggregate. So, the concentration of cryoprotectant had an influence on the size of the nanoparticles due to the low concentration of cryoprotectant might not be enough to protect the particles from the aggregation occurred [236, 237]. Hence, Glu at all concentrations could protect the aggregation while the protection of Tre was concentration dependence. At 5% w/v Tre, the larger size obtained was a result of insufficiency of cryoprotectant to prevent the particles from sticking together during the removal of water in freeze drying process. Therefore, all studied concentrations of Glu and Tre at 7.5 and 10 %w/v as cryoprotectant could be used to maintain the freeze dried nanoparticle after storage for 3 months. Additionly, the optimum concentrations of CG, HY and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles.
Figure 69  Effect of different concentrations of Glu and Tre on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C (A) pH, (B) zeta potential and (C) particle size (mean±S.D., n=3) (CG 35 0.125, HY 30 0.200 %w/v and BSA 1.5 mg/mL)
4.6.6.2 Effect of different concentrations of Glu and trehalose (Tre) on % encapsulation efficiency (% EE) and loading efficiency (% LE)

Effect of different concentrations and types of cryoprotectant on stability, % EE and %LE of the nanoparticles kept in dried form at 4 °C is shown in Figure 70. The %EE and %LE of all concentrations and types of cryoprotectant were around 35-48% and 17-25%, respectively. The %EE and %LE at initial time did not significantly differ except for Glu 7.5%w/v was higher than the others. The % EE & %LE of the nanoparticles that used Glu concentration of 7.5 and 10.0 %w/v was decreased after 3 months of storage while the opposite was found for Tre at the same concentrations. The result may be due to the higher hygroscopic property of Glu than Tre [199]and the absorbed water may dissolve some BSA from the nanoparticle resulting in the reduction in %EE and %LE.
Figure 70  Effect of different concentrations of Glu and Tre on stability of BSA-loaded CG-HY_HY NP kept in dried form at 4 °C, (A) encapsulation efficiency (EE) and (B) loading efficiency (LE) (mean±S.D., n=3) (CG 35 0.125 %w/v, HY 30 0.200 %w/v and BSA 1.5 mg/mL)
4.7 Cytotoxicity test using Caco-2 cells

4.7.1 Effect of different concentrations and molecular weights of chitosan glutamate on relative cell viability

The MTT assay was carried out to evaluate the cytotoxic activity in this fabrication, and Caco-2 cell lines representing intestinal epithelium was used. The studied factors were 3 molecular weights and 6 concentrations of CG were investigated for % cell viability. The pH 6.0 and 7.4 were chosen to simulate pH of intestinal fluid and body fluid, respectively [239-241]. Dextran sulphate sodium salt (0.1% w/v) and SDS (0.1% w/v) were used as positive and negative controls, respectively. Figure 71 shows the effect of incubation at pH 6 and 7.4 for 2 h of different molecular weights and concentrations of CG on relative cell viability. The half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC50) was used to determine the cell viability. At pH 6.0, all factors gave the % cell viability lower than 50% (Figure 71A). At the same concentration, the result was different depending on the molecular weight of CG. The % cell viability of CG 35 and CG 200 at the lowest concentration (0.01%w/v) was higher than CG 45. The result of CG 45 showed concentration independence while CG 35 and CG 200 behaved differently. The higher pH caused the significant increase in cell (%p<0.05) viability at the concentration below 0.05%w/v for CG 35 and CG 45 while at higher MW(CG 200) significant change was reported at the concentration below 0.025 %w/v as shown in Figure 71B. The viability was higher than 50% at these concentrations. The other concentrations of all molecular weights of CG were lower than 50 %. These results indicated that the pH of CG had an impact on the cell viability. The dead cell was a result of the interaction between positively charged chitosan and the negatively charged cell membrane, leading to the death of cell line [239, 242, 243]. The CG at pH 6.0 which is lower than pKa of chitosan (6.5) tended to ionize into a greater extent giving the higher positive charge than CG at pH 7.4 and hence the higher effect to the cell [22, 23]. Figure 72 shows the effect of incubation time for 24 h at pH 6.0 and 7.4 of different molecular weight and concentration of CG, on the % cell viability. As shown in Figure 72A, the pH of samples was adjusted at 6.0, the longer time of incubation, the lower cell viability was reported. The % cell viability was not different in all concentrations and molecular weights of CG, and the viability was less than
50% for all formulations. Figure 72B shows the increase in pH caused the significant increase in cell viability. At the concentration lower than 0.05% w/v showed higher than 50% cell viability. In general, the longer time of incubation at pH 7.4 did not have the effect on cell viability compared with pH 6.0. Therefore, at pH 7.4 the chitosan salt had less effect to the cell stability such that the longer incubation time (24 h) showed slight change [244]. The factor of pH seems to have an influence on the cell viability rather than the incubation time. The %cell viability was molecular weight and concentration dependence.
Figure 71  Effect of different concentrations and molecular weights of CG on relative cell viability incubated for 2 h, pH of sample adjusted at 6.0 (A) and 7.4 (B) (mean±S.D., n=3)
Figure 72 Effect of different concentrations and molecular weights of CG on relative cell viability incubated for 24 h, pH of sample adjusted at 6.0 (A) and 7.4 (B). (mean±S.D., n=3)
4.7.2 Effect of different concentrations and hydrolysis time of hydrolyzed shellac on relative cell viability

The MTT assay was also carried out to evaluate the cytotoxic activity of hydrolyzed shellac. Effect of different concentrations of hydrolyzed shellac and hydrolysis times on relative cell viability, investigated at pH 6 and 7.4, and the cell line was incubated with the HY for 2 h as shown in Figure 73. The HY 0 (native) had the cell viability over 50% at all concentrations except at 0.25%w/v. The higher concentration, the lower cell viability was found for HY 15, HY 30 and HY 45. The over 50% cell viability for HY 15, HY 30 and HY 45 were reported only at the concentration of 0.01 %w/v while the others had the lower %cell viability. In general, the longer hydrolysis time, the lower cell viability was obtained. It may be due to the higher negative charge of longer hydrolysis time which was in agreement with the report of Rathore & Ghosh [242]. They demonstrated that the enhancement of cytotoxicity in various charged material is highly dependent on the surface charged, and the order of ranking of cytotoxicity were positive charge>negative charge>neutral. Therefore, the longer hydrolysis time and the higher concentration showed the higher negative charge causing the lower cell viability. One of reasons may be explained the result that the aggregation was occurred after treating with HY 0 on the cell line for 2 h. The aggregating HY 0 caused false positive of cell viability that it was higher than 50% at low concentration of HY 0.

As shown in Figure 73B, the concentrations at 0.01-0.1 %w/v of all hydrolysis times displayed cell viability over 50%. The increased concentration, the lower cell viability was obtained for all shellacs. The result correlated with the longer hydrolysis time that increased the negative charge. The increased pH caused the increased cell viability significantly (p<0.05) for all hydrolyzed shellacs. Because at pH 7.4 is a normal pH body fluid that cell can survive better than pH 6.0 [240, 241]. However, the longer incubation time for 24 h the reduction in cell viability for all hydrolyzed shellacs was reported as shown in Figure 74. The incubation time at pH 7.4 had an effect on the cell viability which was different from the result of CG. All the hydrolyzed shellacs showed the very low viability. However, at 0.25%w/v showed the highest viability which was in contrast with the effect of higher negative
of higher concentration. The result could be the false positive due to the aggregation occurred.

Figure 73 Effect of different concentrations of hydrolyzed shellac and hydrolysis time on relative cell viability incubated for 2 h, pH of sample adjusted at 6.0 (A) and 7.4 (B). (mean±S.D., n=3)
In conclusion, the %cell viability was depended on concentration, pH, hydrolysis time and incubation time.

Figure 74 Effect of different concentrations of hydrolyzed shellac and hydrolysis time on relative cell viability, pH of sample adjusted at 7.4 and incubated for 24 h. (mean±S.D., n=3)

4.7.3 Effect of different concentrations of BSA-loaded chitosan-shellac nanoparticles on relative cell viability

Effect of different concentrations of BSA-loaded CG-HY_HY NP on relative cell viability which was adjusted to pH 6.0 and incubation time for 2 hours is shown in Figure 75. All concentrations of nanoparticles displayed lower than 50% of cell viability, and it had a trend to increase with the increase in concentration. However, the opposite result was reported at pH 7.4 as shown in Figure 75B. The % cell viability showed 100% at the lowest concentration of nanoparticles, and it had a trend to decrease when the concentrations of nanoparticles were increased. Because at the high concentration of the nanoparticle had more amount of CG 35 and HY 30 which gave a high cytotoxicity causing cell dead. This result could prove that the pH
and concentration played an influence on the viability of cell. The nanoparticle formation by preparing at low concentration of HY and CG could be used to encapsulate BSA and gave the higher % cell viability than other ones.

Figure 75 Effect of different concentrations of BSA-loaded CG-HY_HY NP on relative cell viability incubation time for 2 h, adjusted pH at 6.0 (A) and 7.4 (B) (mean±S.D., n=3)
4.7.4 \( IC_{50} \) of different molecular weights of CG and hydrolysis time of HY

In addition, the half maximal inhibitory concentration (\( IC_{50} \)) is one of the important values for the measurement of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measurement indicates that the amount of a particular drug or substance is needed to inhibit a given biological process by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or \( IC_{50} \)). The \( IC_{50} \) of samples (CG and Hy) is shown in Table 23. The \( IC_{50} \) of CG 35 and CG 45 were equal and the CG 200 showed the lowest IC50 value than the other two indicated the highest cytotoxicity with the cell line. This result was in agreement with Opanasopit et al.[239] which reported the lower IC50 of the higher molecular weights of CG. In addition, Mao et al reported that the cytotoxicity of PEGylated trimethyl chitosan (TMC) copolymers increased with increasing MW [243].

In general, the \( IC_{50} \) of HY had a tendency to decrease when the hydrolysis time was longer. The \( IC_{50} \) of HY 0 exhibited the higher value than the others. This result indicated that the higher amount of negative charge of hydrolyzed shellac resulted from the longer hydrolysis process leading to the dead cell. Hence, the anion of shellac was able to attach to the surface of cell line, and leading to dead cell. In comparison with all three hydrolysis times, HY 30 had the highest \( IC_{50} \). In addition all shellacs had an \( IC_{50} \) higher than CG indicated that the cytotoxicity of HY on the cell line was lower than CG.

Table 23 \( IC_{50} \) of samples (mean±S.D., n=3)

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC 50 (% w/v)±SD</th>
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</thead>
<tbody>
<tr>
<td>CG 35</td>
<td>0.057±0.035</td>
</tr>
<tr>
<td>CG 45</td>
<td>0.057±0.023</td>
</tr>
<tr>
<td>CG 200</td>
<td>0.042±0.009</td>
</tr>
<tr>
<td>HY 0</td>
<td>0.208±0.031</td>
</tr>
<tr>
<td>HY 15</td>
<td>0.158±0.027</td>
</tr>
<tr>
<td>HY 30</td>
<td>0.163±0.072</td>
</tr>
<tr>
<td>HY 45</td>
<td>0.148±0.044</td>
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</tbody>
</table>
4.8 Uptake study of nanoparticles

Uptake study is a one parameter that has an importance with a new formulation of micro or nanoparticles. Figure 76 shows the effect of % initial loading of FITC-BSA uptake on the concentration of FITC-BSA after diluting with HBSS. The nanoparticle uptake was also dependent on the incubation time [245]. In comparison with the incubation time between 4h and 6 h of FITC-BSA NP the 4h-incubation time had more % initial loading than 6h for all concentrations of FITC-BSA. Davda & Labhasetwar reported that the rapid uptake of nanoparticle was observed in the first 30 min of contact, and following by gradual increase in cellular uptake in 1 h [245] and it correlated with the result of Ma & Lim [189].

Figure 76 Effect of the uptake FITC-BSA on % initial loading by Caco-2 cell mono layers (mean±S.D., n=3)

In addition, the uptake of nanoparticles also depended on the concentration that increased with the increase in the concentration of FITC-BSA NP. The result was agreement with Ma & Lim [189] that the uptake of nanoparticle was concentration
dependent, the 2h uptake increasing by 2.07-fold, from 35.99 to 74.36 g/mg, when the loading concentration was increased. In solution form of FITC-BSA, % initial loading was significantly lower than all concentrations of the nanoparticle form at the same concentration. Therefore, the efficiency of uptake of nanoparticles was significantly higher than solution form through the mechanism of transcellular uptake [12, 189]. Therefore, binding of chitosan from FITC-BSA NP to the cell membrane could be more efficient on the cell uptake than only using FITC-BSA solution [189]. Glucose as a cryoprotectant, in general, had a lower uptake than 4h-FITC-BSA NP except at the lowest concentration. Glucose is probably interrupted the process of the uptake of nanoparticles through the cell, hence the lower uptake was found [246].

Quantification of cellular uptake in term of µg is shown in Figure 77. The uptake pattern of every ratio between FITC-BSA: HBSS was similar to the graph of % initial loading. However, amount of cellular uptake in all formulations were displayed in a very low quantification including the FITC-BSA NP. It may be due to the large molecular weight of BSA that it was more difficult to permeate through the cell [247].

Figure 77 Effect of the uptake FITC-BSA on quantification of cellular uptake by Caco-2 cell monolayers (mean±S.D., n=3)
Figure 78 Effect of the uptake FITC-BSA on amount (µg)/amount of cellular protein (µg) by Caco-2 cell mono layers, (A) Colloidal form and (B) solution form (mean±S.D., n=3)
The graph of amount of uptake was recalculated to correct the error due to the unequal starting of seed cell and each formulation was compared with the amount of FITC-BSA as shown in Figure 78A. The uptakes of FITC-BSA in all formulations were increased until ratio of FITC-BSA: HBSS was 60:40. At ratio of FITC-BSA NP: HBSS was 80:20 and 100:0 displaying the uptake of nanoparticle through the cell had almost no difference. It may be due to the limited cellular uptake at these ratios [247]. Therefore, the ratio of FITC-BSA NP: HBSS at 60:40 displayed the maximum uptake of nanoparticle to permeate through the cell. The uptake of FITC-BSA solution is shown in Figure 78B and the similar pattern was reported. Nevertheless, the amount of uptake of FITC-BSA solution was significantly lower than the amount of FITC-BSA nanoparticle.

Hence, FITC-BSA nanoparticle could enhance the cellular uptake significantly which was significant higher than the FITC-BSA solution over 2000-fold.
4.9 Stability of protein using gel electrophoresis

Gel electrophoresis test was used to investigate the stability of BSA. As shown in Figure 79 is the comparison between supernatant and precipitate of BSA-loaded chitosan-shellac nanoparticles after centrifugation. The BSA solution was used for the control as shown in Figure 79A, and blank-loaded CG HY NP in the formulation represented in Figure 79C. The gel electrophoresis of supernatant and precipitate which is shown in Figure 79B and 79D that did not significantly differ from the control. The result suggested that the nanoparticle formation by polyelectrolyte complex between CG and HY did not change the structure of protein [238].

![Figure 79 Gel electrophoresis for stability test of (A) BSA solution (control), (B) supernatant of BSA=1.5 mg/mL, (C) blank-loaded CG HY NP and (D) precipitate of BSA-loaded CG-HY_HY NP BSA=1.5 mg/mL (CG 35 = 0.125 %w/v, HY 30 = 0.200 %w/v)
The effects of freeze drying process and cryoprotectants on the stability of protein were also fabricated with gel electrophoresis as shown in Figure 80. All formulations displayed brand of BSA protein as same as the control. So, the structure of protein did not change due to the cryoprotectants such as trehalose and glucose including the freeze drying process. The result suggested that the cryoprotectant and freeze drying process did not have an influence on a protein structure [238].

![Figure 80](image)

Figure 80 Gel electrophoresis for stability test of BSA-loaded CG-HY_HY NP (A) BSA solution (control), (B) No cryoprotectant, (C) using trehalose as a cryoprotectant and (D) using glucose

The time of storage was reported to have an effect on the protein loaded nanoparticle [199]. The storage time might cause a deformation of protein structure. Figure 81 shows the effect of storage time and types of cryoprotectant on the stability of protein structure by running gel electrophoresis. All patterns of protein brand as displayed in the Figure 81 were in a similarity. The result indicated that the storage temperature at 4 °C for 3 months and both types of cryoprotectant did not cause the deformation of protein structure.
Figure 81  Gel electrophoresis for stability test of BSA-loaded CG-HY_HY NP were kept at 4 °C for 3 month (A) BSA solution, (B) using glucose as a cryoprotectant, (C) using trehalose as a cryoprotectant and (D) No cryoprotectant

In vitro release, many researchers have concerned with the stability of protein structure after it was released from the loaded-nanoparticle to the medium test. Figure 82 shows the effect of each medium type and sampling time on the stability of protein structure. The ionic strength and pH of each medium probably affected the protein deformation [248]. The release of protein in simulated gastric and intestinal fluid at several pH and time was carried out. The protein was sampling at certain time i.e., 1hr in SGF pH 1.2, 3 hr in SIF pH 6.8; 6, 12 and 24 hr in SIF pH 7.4. All the protein bands were similar to the control BSA. Therefore, the test media did not cause the change in the protein structure.
Figure 82 Gel electrophoresis for stability test of BSA release from the BSA-loaded CG-HY_HY NP at various times; (A) 1h in SGF pH 1.2, (B) 3h in SIF pH 6.8, (C) 6h in SIF pH 7.4, (D) 12h in SIF pH 7.4, (E) 24h in SIF pH 7.4 and (F) BSA solution
Nanoparticulate delivery systems have been widely investigated in the pharmaceutical industry due to the ability of controlled release of peptide drugs, the protection from degradation in the GI tract and the enhancement of transmucosal transport leading to an improvement of bioavailability. In this study, the ionic cross-linking method was used to form the BSA loaded nanoparticle. This method is required the interaction between polyelectrolyte property of positively and negatively charged materials. In general, chitosan and tripolyphosphate possesses positive and negative charges are used widely. The attempt in this study was to use the two biopolymers possessing polyelectrolyte for the formation of nanoparticles. The positive charged salt from chitosan glutamate was used instead of chitosan base due to the water solubility and the higher encapsulation. The negative charge was from native shellac including various times of hydrolyzed shellac to give a smaller size and higher polyanion instead of tripolyphosphate. Initially, the native shellac and CG were used to form nanoparticle for encapsulating BSA. Depending on the concentrations of CG, HY0 and BSA, there were three physical states; solution, nanoparticle and aggregation. The optimum concentrations of CG, HY0 and BSA contributed to the balance between the attractive force and repulsive force from the anionic and cationic molecules leading to the formation of the nanoparticles. The particle size of the colloid was in the 100-300 nm nanometer size range, and the zeta potentials of all nanoparticles displayed a positive charge. The morphology characteristic of the BSA-loaded chitosan-shellac nanoparticles was proved by using TEM, and spherical shape of the nanoparticles was observed. The FT-IR and DSC were used to confirm that the nanoparticle was formed by using CG and HY0 for the encapsulation of BSA. The change in spectra of FT-IR and DSC thermogram proved that the formation of nanoparticles could be achieved by the application of two natural oppositely charged polymers for the encapsulation of BSA. The concentration of CG, HY0 and BSA had an influence on the EE and LE, and associated with the zeta potential. The EE and LE were in the range of 11-67% and 7-45 %, respectively. For in vitro BSA release, the nanoparticles showed the immediate release of BSA from
the matrix of all systems, and the amount of release was between 64.3-78.1% significantly depending on the concentrations of CG. After 30 minutes, a gradual and the slow release was reported. The immediate release was due to the part of adsorbed BSA at the surface and the competitiveness between phosphate ion and the anion of BSA at the binding sites.

In order to improve the nanoparticle formation, the longer hydrolysis time of shellac was used to investigate. The various times of hydrolyzed shellacs were prepared because the assumption of smaller size and higher anion of hydrolyzed shellac could be applied better as the polyanion to form the nanoparticles. The physical morphology of the longer hydrolysis time of shellac was stickier than a short time of hydrolyzed and native shellac. The decrease in the beginning melting temperature of prolonged hydrolysis time was shown by using hot-stage microscopy indicating the breaking of the ester bonds of shellac giving the strength of bonds differently. The result was correlated with the increase in AV indicating the breaking of the esters bond of shellac giving the free carboxyl groups during the hydrolysis process. In addition, the higher free carboxyl group indicating by the higher polar force and total surface free energy which was shown in extended hydrolysis time. DTA, FTIR, XRD and Simultaneous XRD–DSC measurement were used to prove a different characteristic between native shellac and other hydrolyzed shellacs. DTA displayed the endothermic peaks around 40-100 °C attributing to the elimination of absorbed water and the melting endothermic peak of all shellacs at the similar range of temperature. The FT-IR spectra of shellac and hydrolyzed shellac showed the characteristic peak of carbonyl stretching at 1,716 cm⁻¹ and C–O stretching at 1,255 cm⁻¹. XRD and Simultaneous XRD–DSC measurement also could not clearly separate the pattern of all hydrolyzed shellacs. In addition, DTA and XRD and Simultaneous XRD and DSC measurement were also used to confirm the interaction of each material for the formation of nanoparticle indicating that the nanoparticle could be formed by polyelectrolytes complexes of CG, HY and BSA. The different molecular weights of chitosan have an influence on the nanoparticle displayed. The longer chain of high molecular weight of chitosan caused the increased viscosity of circumstance then it can prevent the particles to come closer until it was aggregated. Varying of hydrolysis times of shellac had an effect on, zeta potential and particle size
of the nanoparticle. The increase in hydrolysis time of shellac did not result in the reduction of zeta potential but the opposite result was noted. This result suggested that the increase in the zeta potential with the increase in hydrolysis time of shellac attributing to the increase in the unoccupied amine group of CG due to the competitiveness of deprotonated carboxylic groups between shellac and BSA. The particle size of the colloid was in the 105-250 nanometer size range. The lower % EE and % LE with the increase in hydrolysis time of shellac was also in accordance with the increase in the zeta potential indicating the unoccupied amine of chitosan and hence lower %EE and %LE. The effect of concentrations of CG and BSA on the formation of nanoparticle correlated with the preliminary study. pH is the one important factor that affected the formation of the nanoparticles. The increase in the pH led to the reduction in the degree of protonation of the chitosan, and hence the decrease in the zeta potential. This caused the decrease in the electrostatic repulsion force between the particles leading to the aggregated particles. The result of %EE and %LE with the increase in pH was in accordance with the reduction in the zeta potential confirming that there was an interaction between the positive charge of CG and the negative charge of BSA. In addition, if pH of the nanoparticle was adjusted too high, the aggregation state will be occurred. For BSA release in SGF pH 2.0, the nanoparticle cannot protect BSA in SGF pH 2.0, and the immediate release of BSA from the matrix was shown. Hence, the enteric coated capsule was used in this study to protect the nanoparticles from the hazard environment in GI tract. The release of BSA from the nanoparticle was not observed in 2 h in all formulations. The various concentration of shellac and hydrolysis time did not significantly affect BSA release in SIF pH 6.8 and pH 7.4. The cumulative release was depended on %EE, particle size, molecular weight and concentration of CG. It was found that the release of CG35 was lower than CG45. The influence of molecular weight of polymer on the mechanism of drug or protein release from nanoparticles has not yet been clarified. The CG at the concentration of 0.125 %w/v displayed the lowest release than the other concentrations. It may be due to the larger size of the nanoparticle attributing to the larger diffusion path length for the BSA and the lower contact surface area of the larger particles with the dissolution medium.
In addition of conditions as described above, the major obstacle that limits the use of the nanoparticles is due to the instability. The instability is frequently observed when the nanoparticles are stored for an extended period. The instability of the nanoparticle was reported and the aggregation was seen when it was kept for a long time at room temperature and 4°C. The aggregation was due to the reduction in zeta potential. The dried form of the nanoparticle showed the improved stability with the addition of trehalose and glucose as a cryoprotectant. The physical properties of the dried nanoparticle after 3 months of storage at 4°C were not significantly different for both types of cryoprotectants. Hence, the stabilized nanoparticle could be obtained when it was prepared in dried powder form with the aid of cryoprotectant. Additionally, gel electrophoresis was used to evaluate the stability of conformation of BSA. The brand of protein in all formulations as shown in gel electrophoresis did not greatly differ from the BSA control. Hence, the effect of freeze drying process, cryoprotectants, the time of storage, and medium for in vitro release did not affect on the conformation of BSA.

The MTT assay was carried out to evaluate the cytotoxic activity in this study and Caco-2 cell lines representing intestinal epithelium was used. The adjusted pH of CG played an importance role with cell viability that at pH 6.0, the cells showed the lower viability than pH 7.4. The CG at pH 6.0 which is lower than pKa of chitosan (6.5) tended to ionize into a greater extent giving the higher positive charge than CG at pH 7.4. The dead cell was a result of the interaction between positively charged chitosan and the negatively charged cell membrane, leading to the death of cell line. The different incubation time at the same pH had a slightly effect on the viability of the cells. The high molecular weight of CG caused cell dead more than low molecular weight as described in term of IC50. The HY was also tested with MTT assay. The varying in incubation time and pH had an influence on the cell viability that low and high pH and incubation time caused higher cell dead. The hydrolyzed shellac with the longer hydrolysis time showed the lower IC50 indicated that it had higher cytotoxicity than native shellac and short time of hydrolysis shellac. Hence, the positive charge and negative charge of CG and HY respectively had a great effect on cell dead. The lowest concentration of the nanoparticles gave the high % cell viability over 50% at pH 7.4 and incubated for 2 h, and it had a trend to
decrease when the concentrations were enhanced. Hence, at the same concentration of the positive charged CG had higher cytotoxicity than the negative charged HY and the low concentration of NP could be used for the development of oral protein delivery because of the lowest cytotoxicity.

The uptake study of nanoparticle was investigated comparing with the solution using FITC-BSA for a protein model. The uptake of the FITC-BSA NP was significantly higher than FITC-BSA solution for all concentrations. The increase in incubation time from 4 h to 6 h did not affect the increase in uptake of FITC-BSA NP, but it was lower than in high ratio of FITC-BSA NP. The ratio of FITC-BSA NP: HBSS at 60:40 was displayed the maximum concentration of the uptake of nanoparticle permeate through the cell when the amount of the Caco-2 cell was calculated based on an equal in any ratio of FITC-BSA NP. It may be due to the limited cellular uptake at the high ratios.

In summary, the nanoparticles could be prepared by ionic cross-linking between CG and HY to encapsulate BSA. The optimum concentration of each material, various times of hydrolyzed shellac and pH played an important role in the formation of the nanoparticle. The enteric coated capsule could protect the nanoparticle in SGF pH 2.0 medium and was gradually released in SIF pH 6.8 and 7.4. The preparation of protein loaded nanoparticles could prove to uptake through the Caco-2 cell into a significantly higher extent than in solution form. The nanoparticle could be stabilized for more than 3 months at 4°C in a dried form with the aid of glucose and trehalose as a cryoprotectant. Hence, HY could be applied as natural polyanion for polyelectrolyte complex. CG and HY, the natural polymers, are potentially useful polymers for the nanoparticulate carrier as protein and drug delivery systems. The dried loaded protein nanoparticle could be proved for the formulation of oral protein delivery system.
BIBLIOGRAPHY


APPENDIX
**Acid value**

Acid value (AV) of each sample was calculated using the following formula.

\[
AV = \frac{V_{NaOH} \times N_{NaOH} \times 56.11}{W_{sample}}
\]

where

- AV is acid value (mg KOH/g sample),
- \(V_{NaOH}\) is the amount of NaOH consumed at the equivalent point (ml),
- \(N_{NaOH}\) is the molarity concentration of NaOH (M),
- 56.11 is the molecular weight of KOH, and
- \(W_{sample}\) is the amount of NaOH (g)

**Example**

1.782 g of Hy 0 utilized 27.14 mL of 0.0989 M NaOH to neutralize the acid.

\[
AV_{Hy\,0} = \frac{27.14 \times 0.0989 \times 56.11}{1.782}
\]

\[
AV_{Hy\,0} = 84.52
\]

The AV of Hy 0 was 84.52 mg KOH/g sample
Determination of the amount of BSA

Standard :  BSA solution
Method :  UV spectrophotometry (Lowry method)
Detector :  The UV wavelength at 550 nm
Concentration (mg/mL) :  0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4

Figure 83  Standard curve of BSA solution for encapsulation and loading efficiency study

Figure 84  Schematic of capsule before (A), and after coating with Eudragit® S100 and L100-55 (B)
Figure 85  The effect of HY 0 on cytotoxicity incubated with Caco-2 cells

Figure 86  The effect of HY 15 on cytotoxicity incubated with Caco-2 cells
Figure 87 The effect of HY 30 on cytotoxicity incubated with Caco-2 cells

Figure 88 The effect of HY 45 on cytotoxicity incubated with Caco-2 cells
Figure 89  The effect of CG 35 on cytotoxicity incubated with Caco-2 cells

Figure 90  The effect of CG 45 on cytotoxicity incubated with Caco-2 cells
Figure 91  The effect of CG 200 on cytotoxicity incubated with Caco-2 cells
Determination of the amount of FITC-BSA solution

Standard : FITC-BSA solution
Method : Fluorescence spectrophotometry
Detector : The excitation wavelength at 490 nm
The emission wavelength at 525 nm
Concentration (%w/v) : 0.0025, 0.005, 0.0075, 0.01, 0.0125

Figure 92  standard curve of FITC-BSA solution for cellular uptake study
**Determination of the amount of FITC-BSA NP**

**Standard**: FITC-BSA NP  
**Method**: Fluorescence spectrophotometry  
**Detector**:  
- The excitation wavelength at 490 nm  
- The emission wavelength at 525 nm

**Concentration (μg/mL)**: 100, 200, 300, 400

![Figure 93 standard curve of FITC-BSA NP for cellular uptake study](image)

Equation:  
\[ y = 150.61x \]

\[ R^2 = 0.9757 \]
Determination of the protein content

Standard : BSA solution
Method : UV spectrophotometry (protein assay kit)
Detector : The UV wavelength at 570 nm
Concentration (µg/mL) : 22, 44, 66, 88

Figure 94  Standard curve of BSA solution for protein content in cellular uptake study
Table 24  List of abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>ºC</td>
<td>degree Celsius</td>
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<tr>
<td>AV</td>
<td>acid value</td>
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<tr>
<td>XRD</td>
<td>powder X-ray diffraction</td>
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<tr>
<td>DTA</td>
<td>Differenctial Thermal Analysis</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
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<tr>
<td>IC₅₀</td>
<td>the half maximal inhibitory concentration</td>
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<tr>
<td>FITC</td>
<td>fluorescence isothiocyanate</td>
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<tr>
<td>FITC-BSA NP</td>
<td>fluorescence isothiocyanate-BSA- Nanoparticle</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<tr>
<td>μL</td>
<td>microliter</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
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<td>%</td>
<td>percent</td>
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<td>%v/v</td>
<td>percent volume by volume</td>
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<tr>
<td>%w/v</td>
<td>percent weight by volume</td>
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<tr>
<td>%w/w</td>
<td>percent weight by weight</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>cm²</td>
<td>square centimeter</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CH</td>
<td>chitosan base</td>
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<td>CG</td>
<td>chitosan glutamate</td>
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<td>chitosan glutamate 35 kDa</td>
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<td>HY</td>
<td>shellac</td>
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<td>Symbol</td>
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<tr>
<td>HY 0</td>
<td>hydrolyzed shellac at 0 minutes (native shellac)</td>
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<td>BSA-loaded chitosan-shellac nanoparticles</td>
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<td>Fourier transform infrared</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
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<td>carbon dioxide</td>
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<tr>
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<td>glucose</td>
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<tr>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>s</td>
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<td>sodium hydroxide</td>
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<td>NP</td>
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<td>Symbol</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<td>gram</td>
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<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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BIOGRAPHY

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Publication

Presentation

