Chapter-6

Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film
6.1. Introduction

Currently, fluorescent nanoparticles (NPs) (also known as quantum dots, QDs due to their typical sizes below 10 nm), a young smart member of the nano-material family is gaining attention, especially in the field of optoelectronics, bio-imaging, biomedicines, etc. Unfortunately, application of NPs in food\textsuperscript{1-5} and its packaging materials is still in its infancy. The reason behind this seems to be the raised toxicity concern and environmental hazard of the fluorescent semiconductor QDs, which are based on metallic elements and heavy metals (elements from the periodic groups II-IV, III-V and IV-VI). Alternatively, fluorescent carbon NPs (called fluorescent carbon-dots or FCDs or C-dots or simply CNPs) can become an exciting option, because they are greener for the environment and show aqueous dispersibility, numerous possible applications in nanobiotechnology, and far less toxic to living organisms. Recently, Sk et al.\textsuperscript{6} reported the presence of FCDs in regular carbohydrate-based food items such as bread, jaggery, sugar caramel, corn-flakes and biscuits, and also showed their biocompatibility. This novel discovery alleviated the misapprehension that all fluorescent NPs are toxic and revealed that humans have been consuming fluorescent nano-materials in the form of food caramels for centuries, and thus they can be considered safe.

Recently, procurement of novel C-dots from greener sources such as egg,\textsuperscript{7} orange juice,\textsuperscript{8} banana juice,\textsuperscript{9} soy-milk,\textsuperscript{10} glucose, sucrose, starch,\textsuperscript{11} citric acid,\textsuperscript{12} chitosan,\textsuperscript{13} etc. has been the centre of attraction. Although they are the current state-of-the-art, most of these methods suffer to some degree from certain drawbacks such as the requirement of sophisticated and expensive equipments such as laser beam or microwave or high-power autoclave; neat chemicals as precursors for synthesis, or even multi-steps of operation along with surface passivation to improve the water-solubility and photoluminescent properties of C-dots. Moreover, use of such food/feed or other edible stuffs as raw materials for FCD preparation is expensive for bulk use, and in the long-run would create undue strains on the food resource system and food security scheme. This is a major drawback to the commercial synthesis of NPs from these edible resources, which is
unlikely to be extended in the near future. This motivated us to search for a simple economical technique for the production of FCDs from industrial or agricultural by-products. This is a challenging but worthy concept as the use of waste materials is one of the most attractive options to reduce the raw material cost and also seems benign from an ecological point of view. In this context, very few articles are available. Lu et al.\textsuperscript{14} reported such an endeavor from pomelo peel and the resulting CNP was used for the detection of mercury ions in water. Wang et al.\textsuperscript{15} prepared C-dots from egg-shell membrane and used it for designing a fluorescent probe for glutathione detection. In a closely related work, FCDs were prepared from used coffee grounds,\textsuperscript{16} and their practicality in cell imaging and detection of angiotensin I and insulin was assessed. Most reported C-dots have been prepared for bioimaging which limits their application in other fields. As such, it has become urgent to develop effective routes to create functional C-dots as-well-as expand their applications.\textsuperscript{10} Explicitly the role of FCD in food packaging domain is unexplored to date.

Industrially, mechanical pressing of the oilseeds produces the oil (main product) and the press-cake (by-product). Due to the high quantity of protein, the oil-cake or meal is being used mainly as animal feed. Recently, owing to the high nutritive value of the meal protein, techniques are now being devised to harness it. After the extraction of meal protein, the residual fibrous waste material (protein-spent meal) is discarded and is not extensively used in industries. At present there are very few possibilities for the utilization of this waste; usually the residue is disposed as landfill and hence, is as an “end-of-pipe” waste. In this chapter, the synthesis of green fluorescent quantum-sized carbonaceous NPs from ‘oil-and-protein spent’ rapeseed meal by a facile hydrothermal process has been reported. These NPs are referred as ‘carbogenic’ because of their high oxygen content along with carbon.\textsuperscript{12}

Undeniably, the most active area of food nanoscience research and development is ‘packaging’. This is likely connected to the fact that the public has been shown in some studies to be more willing to embrace nanotechnology in “out-of-food” applications than
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

those in which NPs are directly added to food.\textsuperscript{17} So, the practicability of the synthesized FCDs for making green-fluorescent edible biopackaging material was investigated in this study, thereby expanding the potential application of C-dots. Synthetic plastic packaging is beginning to be replaced by biodegradable ones because of environmental concerns. Films made of carbohydrates and protein are long and empirically been used to make food-grade biodegradable packaging materials. A recent approach to this technology involves the use of vegetable protein from different types of oil-cakes such as cottonseed,\textsuperscript{18} pumpkin seed,\textsuperscript{19} soybean,\textsuperscript{20} and rapeseed/canola.\textsuperscript{21} In this milieu, edible films using rapeseed protein were developed in the current investigation, because this biopolymer can be obtained as value-added product from the under-utilized meal. As such, the study was extended to evaluate the effect of such a nano-additive on the physicochemical properties of rapeseed protein film.

Mostly, the consumers have to rely on holograms and other displays on the packets to segregate authentic products from their duplicate inferior counterparts. Often, fluorescent dyes are incorporated into the packaging to help customers easily detect authentic products (e.g. Erythrosine); however, many of these dyes have been shown to be cytotoxic to a variety of mammalian cell types.\textsuperscript{22} The unique “green” photoluminescence property of the here-in synthesized carbogenic NPs, along with high antioxidative potential and hemocompatibility, offer a solution to this problem. It is envisioned that FCD-incorporated edible films would not only be useful for quality control to ensure that consumers are able to purchase authenticated products, but would also improve the oxidative stability of the produce/commodity. To justify this possibility, further study was conducted to see the effect of FCD-protein composite film on the oxidative shelf-life of an oil sample. For simplicity, the preparative protocol along with the summarization of the complete investigation undertaken in this chapter is depicted pictorially in Fig. 6.1.
6.2. Materials and methods

6.2.1. Chemicals

All solvents and reagents were obtained from E. Merck® (India), of either high-performance liquid chromatography (HPLC) grade or analytical reagent grade, and were used without further purification.

6.2.2. Materials and sample preparation

Cold-pressed rapeseed oil and press-cake were obtained from Assam Khadi & Village Industries Board, Guwahati, India. Press-cake was defatted and then partially detoxified prior to protein extraction according to the procedure mentioned in Chapter-4, and then stored at -20 °C until use.

6.2.3. Protein extraction from detoxified meal and recovery of oil-and-protein spent meal

Aqueous suspension of detoxified meal was prepared with water (30:1 v/w), followed by the addition of 0.0 M NaCl and 0.4% sodium sulfite. The pH of the
suspension was adjusted to 11±0.1 with 1 N NaOH solution; the suspension was mixed (213 x g) for 2 h at 25 °C in the orbital shaker (Sartorius Stedin Biotech, CERTOMAT® IS), followed by centrifugation (SIGMA 3-18K Centrifuge) at 7,513 x g for 20 min at 4 °C. The solid residue (oil-and-protein-spent meal) was vacuum-dried (Lab companion model OV-12, Jeio tech Co., Korea) for 48 h at 40 °C, ground to pass through a sieve having 250 μm pores, and then stored at -20 °C for further use. On the other hand, the protein-rich supernatant was filtered through Whatman filter no. 41 and then ammonium sulfate was added up to 85% saturation. The mixture was kept in an ice bath for 3 h with gentle stirring and then centrifuged at 10,733 x g for 20 min at 4 °C. The obtained protein precipitate was re-dispersed in Milli-Q water (Millipore Water Purification System, Model-Elix, USA), neutralized to pH 7, dialyzed against water at 4 °C and finally freeze-dried, which was subsequently used for making the composite film. Proximate analyses of the spent meal were performed by the methods mentioned in Chapter-2.

6.2.4. Synthesis of C-dots from oil-and-protein spent meal

FCDs were synthesized by hydrothermal carbonization (HTC) of spent meal using the reported protocols\(^8\),\(^23\) with slight modification. In a typical procedure, oil-and-protein spent meal was dispersed in 1N NaOH solution (solvent) such that a definite solvent:meal ratio was attained as per the design matrix. Then the suspension was refluxed in an oil-bath under magnetic stirring, at each of the indicated temperature and heating duration mentioned in the central composite design (CCD). After the reaction is over, the resultant black solution was allowed to cool down and centrifuged at 3220 x g for 10 min to separate out the unreacted residue. The brownish supernatant was washed with dichloromethane to remove the unreacted organic moieties. Subsequently, the aqueous phase was mixed with acetone (water:acetone ratio=1:3 v/v) and the acetonic extract was finally reduced to dryness under vacuum. The residue was dispersed in Milli-Q water, dialyzed against water (using 1 kDa membrane, Himedia, India) and finally dried under vacuum at 40 °C to obtain FCD, whose weight is recorded.
6.2.5. Characterization of C-dots

High resolution transmission electron microscopy, HRTEM (JEOL, JEMCXII) images along with the selected-area electron diffraction (SAED) pattern were obtained at an accelerating voltage of 200 kV, and the sample was prepared by drop casting 2 μl of NP solution (0.25 mg/ml) on a 300 mesh carbon coated copper grids and subsequent air drying before analysis.

X-ray Diffraction (XRD) measurement was carried out by thin film mode of powdered sample using Rigaku Miniflex model (Japan), operated at 30 kV voltages and a current of 15 mA with Cu Ka radiation source (λ=1.54 Å) at a scan rate of 5º (2θ) min⁻¹ over the range of 10–70º.

Thermogravimetric analysis (TGA) curves were collected on a TG 50 model (Shimadzu, Japan). The samples were combusted under nitrogen flow (10 ml/min, to avoid thermo-oxidative reactions) at the temperatures ranging from 25 to 600 ºC, at a rate of 3 ºC/min.

Fourier transform infrared (FTIR) spectra were obtained on a FTIR Nicolet Magna 5PC spectrometer (Impact-410, Madison, USA), coupled to a PC with Omnic analysis software and having DTGS (Deuterated Triglycine Sulfate) detector and Nernst Filament as the IR light source. The sample was ground with KBr powder to form well-defined pellets for IR measurement, with 32 scans from 4000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹.

Micrograph of the sample was obtained using a JSM-6390LV scanning electron microscope (SEM; JEOL, Japan) at an accelerating voltage of 15 kV. Prior to SEM observation, samples were mounted on stubs with double-sided adhesive tape, followed by coating the samples with a thin layer of gold. For determining the elemental composition and purity, sample was prepared on a carbon-coated copper grid and kept under vacuum desiccation for 3 h before loading them onto a specimen holder. Elemental
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

analysis on single particles was carried out using electron dispersive X-ray spectroscopy (EDX, JSM-6390LV) attachment equipped with SEM.

$^1$H- and $^{13}$C-Nuclear Magnetic Resonance (NMR) spectra were detected at 400 MHz by a JEOL NMR system (Japan), using the inbuilt DELTA ($\delta$) software (version-G4.3.6, Japan) provided by the manufacturer.

Ultraviolet-visible (UV-vis) absorption spectrum of aqueous NP solution (0.1 mg/ml) was recorded on a UV-vis spectrophotometer (CECIL 7400, 7000 Series, Aquarius).

Photoluminescence (PL) spectrum of aqueous NP solution (0.1 mg/ml) was measured on a photoluminescent spectrophotometer (Model LS 55, Perkin Elmer, Singapore PTE Ltd., Singapore). Illumination of the aqueous NP dispersion for detecting its fluorescence property was done inside a UV cabinet (BD-198 model, Test Master, Kolkata, India).

Quantum yield (QY) of C-dots was measured in reference to quinine sulphate in 0.1M H$_2$SO$_4$ (QY = 58% at 354 nm excitation).$^{6,24}$ The formula used for QY is as follows:

$$(QY)_{sm} = (QY)_{st} \times \left[ \frac{(PL \text{ area}/OD)_{sm}}{(PL \text{ area}/OD)_{st}} \right] \times \frac{\eta_{sm}^2}{\eta_{st}^2}$$

where Sm indicates the sample, St indicates the standard, $\eta$ is the refractive index of the solvent, and PL area and OD are the fluorescence area and absorbance value, respectively. For aqueous solutions $\eta_{sm}/\eta_{st}=1$ was chosen.$^6$

Antioxidant activity was measured using the modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method.$^{25}$ Briefly, 3 ml of sample solution was mixed with 1 ml of 1 mM methanolic solution of DPPH. The mixture was then vortexed and incubated in the dark at ambient temperature for 30 min. The absorbance was measured at 517 nm in a UV-vis spectrophotometer. In case of FCDs or ascorbic acid (as standard), the amount was varied from 10-180 μg per ml of reaction mixture;$^{26}$ whereas for film samples, 25 mg of each film was dissolved in 3 ml of Milli-Q water.$^{25}$ The DPPH scavenging percentage was calculated using the following formula:
Scavenging activity (%) = \{(A_D - A_S) \times 100\}/A_D \quad (2)

where, $A_D$ and $A_S$ are the absorbance of the DPPH solution and the standard/sample, respectively.

Hemolytic activity assay was performed according to the reported procedure.\textsuperscript{27} Briefly, fresh goat blood from a slaughter-house was collected in a centrifuge tube containing anti-coagulant, tri-sodium citrate (3.2%), and was centrifuged at 3220 x g for 10 min. The supernatant was discarded and only the red blood corpuscles (RBCs or erythrocytes) were collected. RBCs were further washed three times with phosphate buffer solution (PBS, pH=7.4). A 10% (v/v) suspension of erythrocytes in PBS was prepared and 1.9 ml of this erythrocyte solution was placed in a 2 ml centrifuge tube and 0.1 ml of FCD in PBS was added to it. The tubes were then incubated for 1 h at 37 °C. For comparison, Triton X-100 (0.2%) and PBS were taken as the positive and negative controls, respectively. After incubation, the tubes were subjected to centrifugation at 3220 x g for 10 min, and finally absorbance of the supernatant was taken at 570 nm in a UV-visible spectrophotometer.

Cell labeling with FCDs was done by the procedure of Chandra et al.\textsuperscript{28} Erythrocytes enriched fraction was centrifuged twice (3220 x g, 15 min) at 4 °C to remove the residual plasma and buffy coat. RBCs were washed 3 times with PBS (pH 7.4) and re-suspended in the same buffer to make a cell concentration of ≈10% (w/v) as stock. Then, cell labeling was carried out by mixing different aliquot of the above RBC stock suspension with different volumes of FCD (30.4, 45.5, 60.8 and 75.8 μl) from its stock solution (660 mg/ml) for making up the final concentration of FCDs as 20, 30, 40 and 50 mg/ml by addition of PBS in each set of experiment. Afterward, the erythrocytes were incubated for 4 h at room temperature, centrifuged (3220 x g, 15 min) at 4 °C and then washed thrice with PBS. Finally, 20 μl of the cell suspension were taken and used to prepare a smear on glass slides, which was viewed under a fluorescent microscope (LEICA DM 3000, Power: ebq 50 qe, USA) attached with a LEICA DFC 450C camera.
6.2.6. Preparation of rapeseed protein-FCD composite film and its application for oil packaging

Rapeseed protein film solution was prepared by using the methodology of Cho and Rhee.\textsuperscript{20} Freeze-dried rapeseed protein powder (8 g) and glycerol (4 g) were dissolved in 100 ml of Milli-Q water. The suspension was heated on a hot-plate magnetic stirrer for 10 min at 80 °C until a homogeneous clear solution was obtained. FCDs (20 or 30 mg) were added to the mixture and the blending was further continued for 15 min at 80 °C. The mixture (40 ml each) was cast onto glass petriplates and then dried in an oven at 35 °C for 48 h. Films were conditioned in an environmental chamber (Plant growth chamber HB 303DH, K&K Scientific Supplier, India) set at 25 °C with 50% relative humidity (RH) for 48 h. The composite films with 20 and 30 mg of FCDs per 100 ml of film-forming solution were accordingly coded as F1 and F2, respectively. Film without FCDs served as control and was designated as F0.

Films were drawn into small rectangular sachets/pouches (5 cm x 3.5 cm), each of which was filled with 4 ml of cold-pressed rapeseed oil, and then sealed after the headspace had been flushed with nitrogen gas. Oil containing sachets were stored for 28 days in the environmental chamber (25 °C, 50% RH), and the oil samples were analyzed after every 7 days of interval. Oil samples packed in F0, F1 and F2 were accordingly labeled as S0 (control), S1 and S2, respectively.

6.2.7. Characterization of the composite films

Film thickness was measured at 5 random positions with a micrometer (Mitutoyo Corp., Japan). The mean thickness was used to calculate the mechanical and barrier properties of film.

For moisture content determination, film samples were weighed into aluminum pans and dried at 105 °C in an oven for 24 h (until the equilibrium weight). The weight loss of the sample was determined, from which the moisture content was calculated.
Water vapour permeability (WVP) was determined gravimetrically following the standard method of the American Society for Testing and Materials (ASTM).\textsuperscript{25} Conditioned film samples were sealed to glass cups (4.7 cm diameter) containing water. The film-covered cups were placed in the environmental chamber (25 °C, 75% RH). Cups were weighed periodically using an analytical balance (Denver Instrument, Bohemia, USA), until steady state was reached (±0.0001 g). Once the steady state was reached, water vapour transmission rate (WVTR, ng/m\(^2\) s) of the film (Eq. 3) was determined from the slope obtained from the regression analysis of moisture weight gain (\(\Delta w\)) transferred through the film area (A) during a definite time (\(\Delta t\)). WVTR was then used to calculate WVP using Eq. (4).

\[
\text{WVTR}=\frac{\Delta w}{A(\Delta t)} \tag{3}
\]

\[
\text{WVP}=\text{WVTR}(x/\Delta p) \tag{4}
\]

where \(x\) is the average thickness of the film and \(\Delta p\) is the partial water vapor pressure between the two sides of the film (i.e., 3167-2385 = 782 Pa)\textsuperscript{29}

Oxygen permeability of the films was determined by the wet chemical procedure of Ayranci and Tunc.\textsuperscript{30} Tensile strength (TS, in MPa) and Elongation at break (EB, %) of the film was measured according to ASTM D-882-91.\textsuperscript{31} For evaluating puncture strength (given as force in Newton (N)), film was punctured with a 2 mm probe in a Texture Analyzer (TA HD Plus, Stable Micro Systems, UK).

Color intensity of the film was measured using a Hunter Lab Colorimeter (Ultrascan, VIS-Hunter Associates Lab., USA), fitted with a large area port (2.5 cm diameter aperture). The instrument (including 65°/0° geometry, D25 optical sensor, 10° observer, specular light) was calibrated using white and black reference tiles provided by the manufacturer.

For determining opacity, film absorbance was measured at 600 nm using UV-vis spectrophotometer.\textsuperscript{25} The sample was cut into a rectangle piece and directly placed in
spectrophotometer test cell, using an empty test cell as the reference. The opacity index of the film was calculated by following equation

\[
\text{Opacity} = \frac{\text{Abs}_{600}}{x}
\]

(5)

where \( \text{Abs}_{600} \) is the absorbance at 600 nm and \( x \) is the average film thickness (mm).

6.2.8. Analytical tests for oil sample

Free fatty acid (FFA) and peroxide value (PV) were determined according to the method of Chaijan et al.\(^3^2\) and expressed as g/100 g lipid and milliequivalents (meq) of free iodine/kg of lipid, respectively.

Conjugated diene (CD) was estimated from the absorbance value at 233 nm using cyclohexane as the solvent blank.\(^3^3\)

For thiobarbituric acid-reactive substances (TBARS) assay,\(^3^2,3^4,3^5\) oil sample (0.5 g) was mixed with 2.5 ml of a solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25N HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min to develop a pink colour, cooled under running tap water and then centrifuged (3600g) at 25 °C for 20 min. Absorbance value of the supernatant at 532 nm was expressed as the result (\( A_{532} \)).

6.2.9. Statistical analysis

Applying Response surface methodology (RSM) with a \((2)^3\)-CCD matrix, hydrothermal carbonization (HTC) was optimized with 3 combinations of independent variables, namely time (\( X_1 \)), temperature (\( X_2 \)) and solvent:meal ratio (\( X_3 \)). The yield of FCD (\( Y \)) was considered as the response. To have an idea of the amount of NPs which can be extracted from the spent meal, we analyzed the amount of particles obtained from 1 g spent meal under each experimental run. The selected variables were coded at five levels (−1.682, 1, 0, 1, and +1.682) (Table 6.1), and their real values were chosen based on preliminary experiments. The variables were coded according to the following equation:
\[ x_i = \frac{X_i - X_0}{\Delta X_i} \quad (6) \]

where \( x_i \) is the dimensionless coded value of an independent variable, \( X_i \) is the real value of an independent variable, \( X_0 \) is the real value of an independent variable \((X_i)\) at the centre point and \( \Delta X_i \) is the step change value. The complete \((2)^3\)-CCD matrix consisted of 20 experimental runs, including six replications at the centre points (0, 0, 0) to estimate the pure experimental error, and is summarized in (Table 6.2).

The system behavior towards the response was determined by a second-order polynomial equation, based on the equation below:

\[ Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (7) \]

where \( Y \) is the predicted value for the response, \( \beta_0 \) is the offset term, \( \beta_i \) is the linear effect coefficient, \( \beta_{ii} \) is the squared effect coefficient and \( \beta_{ij} \) is the interaction effect. \( x_i x_j \) represents the interaction between different coded values, where \( i \) is one parameter and \( j \) is other.

All analyses were performed in triplicate, unless otherwise indicated, and the mean value was calculated. Separation of means were carried out by Tukey test, using SPSS software (version 16.0, SPSS Inc., Chicago, USA) and considered significantly different at \( p<0.05 \). Analysis of variance (ANOVA) was applied to assess the adequacy (by lack-of-fit test) and statistical significance of the developed model at a confidence level of 95\% (transgression probability, \( p<0.05 \)). Response surface plots for any two independent variables were drawn while fixing the remaining one at coded zero level, for gaining a perspective on the interaction between the variables and how they influence the response, and also to find the optimum condition.
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

Table 6.1 Independent variables and their levels for production of FCD from spent meal by HTC process.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Symbols</th>
<th>Range and levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1.682</td>
</tr>
<tr>
<td>Time (h)</td>
<td>X₁</td>
<td>2.636</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>X₂</td>
<td>126.36</td>
</tr>
<tr>
<td>Solvent:meal ratio (v/w)</td>
<td>X₃</td>
<td>131.8:1</td>
</tr>
</tbody>
</table>

6.3. Results and discussion

The total mass recovery of spent meal was about 61.02±1.7% solid of starting meal (detoxified meal). This value was slightly higher than those reported for yellow mustard and canola meal. C-dots were synthesized by HTC of spent residue, which mainly contains carbohydrates, crude fiber and insoluble structural protein (Table 6.2). These act as the sources of carbon precursors. Till date, very few authors have reported the possible schematic route for the formation of C-dots from carbon precursors, as the formed intermediates and the final material structures are complex and a clear scheme has not yet been reported.

Table 6.2 Proximate composition of spent meal (% dry basis).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean±SD (^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>8.83±0.94</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>18.09±1.07</td>
</tr>
<tr>
<td>Residual oil</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total ash</td>
<td>5.92±0.21</td>
</tr>
<tr>
<td>Moisture content</td>
<td>11.52±0.81</td>
</tr>
<tr>
<td>Total carbohydrate (by difference)</td>
<td>55.63±0.72</td>
</tr>
</tbody>
</table>

\(^8\)Mean ± standard deviation of three replicates
6.3.1. Optimal production of FCD using RSM

The regression equation of the generated model describing the yield (Y) as a function of the uncoded factor levels, is shown in Eq. (8)

\[
Y = 93.19 + 0.38X_1 - 1.09X_2 - 0.03X_3 - 1.47X_1^2 - 1.41X_2^2 \\
- 0.86X_3^2 - 1.78X_1X_2 - 1.21X_1X_3 - 0.08X_2X_3
\]  

(8)

The high coefficient of determination, \(R^2=91.29\%\) (goodness-of-fit test) advocates a good correlation between observed and predicted values, which is also evident from Table 6.3. It also indicates that at least 91\% of the variability in the response could be explained by the derived second-order polynomial equation. Model was highly significant (p<0.05) and p-value for lack-of-fit test was large (p>0.05) (Table 6.4). This in-turn ascertains the validity of the model and is adequate for predicting the response within the conditions investigated here-in.

Fig. 6.2a shows the Pareto chart of the individual, quadratic and interactive effect of the chosen independent variables (\(X_1\), \(X_2\) and \(X_3\)) on the response (Y). The chart includes a vertical line at the critical value for p=0.05. An effect that exceeds the vertical line can be considered significant (p<0.05) and those which falls below this line are taken as non-significant (p>0.05). The need for the use of RSM during NP synthesis is reconfirmed by the Pareto chart; linear term of either temperature or time was found to be non-significant on the yield (p<0.05); whereas the interaction term of time and temperature (\(X_1\)*\(X_2\)), followed by their quadratic terms (\(X_1^2\) and \(X_2^2\)) had the most significant effect. Thus, among the 3 factors, time and temperature of HTC process had significant effect (p<0.05) on the yield; whereas, that of solvent:meal ratio was non-significant (p>0.05). This partly corroborates with the postulations of Ray et al.\(^{23}\)

Generally, lower heating temperature in HTC mandates the need of long processing time. This is in line with the observation of Fig. 6.2b. High temperature seems to produce increased yield in a shorter span of time, which seems judicious for practical
relevance. Likewise, Fig. 6.2c shows that higher yield is attainable by use of long processing time. From Fig. 6.2d, it is clearly apparent that high temperature and large solvent:meal ratio can harbor marked improvement in the yield. Solvent volume ($X_3$) is not a factor frequently studied for its relationship with NP formation; nevertheless, some authors reported the use of high solvent volume during CNP synthesis such as water:carbon soot=600:1 ml/g. Excessively large solvent volume may add to the processing cost of waste water and handling problem during recuperation of NPs.

Table 6.3 Central composite design and response (dependent variable) for FCD production from spent meal by HTC.

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Coded levels of independent variable</th>
<th>Response (Y)</th>
<th>Actual$^1$</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factorial points</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>85.33±0.63$^a$</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>94.30±0.92$^{cd}$</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>87.47±1.11$^a$</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>88.56±0.27$^{ab}$</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>89.02±0.53$^{bc}$</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>92.39±1.89$^c$</td>
</tr>
<tr>
<td>7</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>90.10±0.36$^c$</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
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$^1$Values are mean±standard deviation of n=3 analyses (subjected to Tukey test). Values with the same letter within one column were not statistically different ($p>0.05$).
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

Table 6.4 ANOVA of regression model built for FCD yield.

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SS, sum of squares; df, degree of freedom; MS, mean squares; p, transgression probability

Fig. 6.2 (a) Pareto chart showing the effect of independent variables on the yield of FCD; (b) Yield of FCD as a function of time and temperature; (c) Yield of FCD as a function of time and solvent:meal ratio; (d) Yield of FCD as a function of temperature and solvent:meal ratio.
Based on the regression equation and response surfaces, the predicted optimal condition is 2.6 h of refluxing time, temperature of 179.2 °C and a solvent:meal ratio of 462.8:1 (v/w). In order to make these parameters feasible in experimental run, the optimum parameters were drawn to the nearest round figures\(^4\) of 3 h of heating time, temperature of 180 °C, and solvent:meal ratio of 463:1 (v/w). To verify the predicted values, additional experiments were conducted at the feasible optimum condition. The predicted yield at the optimum condition was 98.39 mg/g dry meal and the real value obtained by experimentation was found to be 95.73±0.9 mg/g dry meal. The closeness between the experimental and predicted values indicates the suitability of the model for prediction and shows a substantial improvement in the process yield, which is important from a commercial point of view.

**6.3.2. Characterization of FCD**

The formation of C-dots was confirmed by TEM measurement (Fig. 6.3a). HRTEM images clearly revealed that the NPs are spherical in shape; their diameters mainly lay in the range of 4.71-10.77 nm with maximum population at 7.66 nm. It can be seen that there are holes or crevices on the surface of C-dots, giving them an appearance of a puffer-like hollow ball (Fig. 6.3b). The average diameter of these surface holes was calculated to be 0.42 nm (approx.). SEM image (Fig. 6.3c) indicates that dried FCD powder is an aggregate of small carbon grains hundreds of nanometers in size. The entities appear to be non-homogeneous in size and shape. This asymmetry is probably due to the effect of drying, because of which the NPs formed clusters of varying sizes and shapes.\(^2\)

The absence of any discernible lattice fringes in HRTEM image suggests an amorphous nature of the as-prepared NPs. Concomitantly, SAED pattern of the C-dots showed diffused rings (Fig. 6.3d), revealing an amorphous carbon phase,\(^7\) which agrees well with the HRTEM analysis. Furthermore, the XRD pattern (Fig. 6.3e) presented a broad hump-like peak near 25.8° which is attributed to amorphous carbon.\(^1\)
Fig. 6.3 (a) TEM image of FCD thus formed; (b) The corresponding HRTEM image of two nanoparticles. The inset shows the pictorial representation of the morphology of one nanoparticle; (c) SEM image of the clusters of nanoparticles obtained after drying; (d) SAED image; (e) XRD pattern; and (f) TGA curve of FCDs.
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

Fig. 6.4 (a) FTIR spectrum; (b) EDX data; (c) $^1$H-NMR; and (d) $^{13}$C-NMR of FCDs.
The thermal behavior of FCDs was investigated by TGA. The thermogram (Fig. 6.3f) exhibited a two-step degradation pattern with the initial degradation (<100 °C) ascribed to the loss of moisture and volatile products, while the final degradation (286.24–358.21 °C) was associated with the decomposition of oxygen-containing groups.41

The FTIR spectrum was acquired to gain further structural insights and surface state of the synthesized FCDs. In this spectrum (Fig. 6.4a), the stretching frequencies observed at 651, 908, 1086, 1233, 1367, 1638, 1697, 2981 and 3421 cm⁻¹ indicate the presence of olefinic C-H (out-of-plane), epoxy ring, C-O or C-O-C, carboxylate group, C-N, C=O, C-H, hydroxyl (-OH) stretch, respectively.9 The different oxygenous functional groups as evident from FTIR, proves that the surface of FCD is partially oxidized. Origin of these functional groups can be ascribed to the degradation of the carbohydrates, crude fibres and residual protein present in the spent-meal through hydrothermal treatment.10,14 Additionally, the presence of these hydrophilic functional groups imparts excellent water solubility to the C-dots without further chemical modification, which in-turn promotes the environmental friendliness of the product.9

EDX data of C-dots (Fig. 6.4b) further confirms the fact that the as-prepared NPs mainly consist of carbon (47.81 wt%) and oxygen (52.18 wt%). EDX spectrum of ‘oil-and-protein’ spent meal, showing its various elemental compositions, is given in Fig. 6.5 for comparison.

![EDX spectrum of ‘oil-and-protein’ spent meal.](image)
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

Fig. 6.6 (a) UV-vis spectrum; (b) PL spectrum of aqueous dispersion of FCDs (0.1 mg/ml) (Excitation at 350 nm); (c) Photographs of aqueous FCD dispersion (0.25 mg/ml) observed under normal light and the same under UV light; (d) gradual bleaching of DPPH solution by FCDs; (e) DPPH scavenging activity of FCDs in comparison to that of ascorbic acid.
Fig. 6.7 Plausible mechanism for the DPPH scavenging activity of FCDs.
NMR spectra highlighted the presence of two different kinds of chemical environments or regions, which are discussed as follows. In the $^1$H-NMR spectrum (Fig. 6.4c), the regions found are: $\delta=1-3$ ppm (for sp$^3$ C-H protons) and $\delta=3-6$ ppm (for protons attached with hydroxyl, ether and carbonyl groups). Also, in the $^{13}$C-NMR spectrum (Fig. 6.4d), similar regions were found; namely, $\delta=20-100$ ppm (for sp$^3$ carbons and for carbons attached with hydroxyl-group or ether linkage) and $\delta=214-215$ ppm (for keto, –C=O carbon atoms). Specifically, the peaks within $\delta=28-33$ ppm depicts the presence of methyl groups in the surface functional moieties and the singlet peak at $\delta=70.67$ ppm indicates the occurrence of carbohydrate units in FCDs.

To explore the optical properties of FCDs, UV-vis absorption (Fig. 6.6a) and PL study were carried out. FCDs showed maximum UV absorption peak at 244.96 nm which likely to have originated from the formation of multiple polyaromatic groups where $\pi-\pi^*$ transitions of the aromatic C-C bond occurs. Most C-dots are excited in the UV region (330-420 nm) and yield photoluminescence in the wavelength range of 400-600 nm depending on the size of C-dots. Fig. 6.6b shows the PL emission peak of FCDs centered at 480.09 nm, with excitation at 350 nm. This is consistent with the literature. From the fundamental as-well-as application view-point, fluorescence is one of the most fascinating behaviors of the C-dots. The aqueous solution of FCDs is bright yellow and transparent in normal light but changes to an intense green colour under UV light (Fig. 6.6c), signifying the NPs to be fluorescent. It is worth noting that the origin of fluorescence from the obtained dispersion could be attributed only to the presence of C-dots because the preparatory ingredients (spent rapeseed meal, water and NaOH) are known to be non-fluorescent materials (i.e. non-emissive under UV light), further confirming the formation of luminescent NPs. QY measured using quinine sulfate as a reference was found to be 9.2%, which is comparable with those of the reported luminescent C-dots. The fundamental mechanism of fluorescence of C-dots is still an open question; however it is thought that the presence of quantum size and different surface trap sites could be the main factors. It is believed that radiative recombination
between electrons and holes (excitons) take place at the particle surface, under the illumination of excitation light, and thus fluorescence emission occurs.\textsuperscript{42}

With the aim of detecting the radical scavenging activity of FCDs, the DPPH assay was performed. As known, a freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades/disappears asSoon-as an antioxidant is added into the medium, resulting in a decrease in absorbance at 517 nm. Interestingly, it was found that unlike other antioxidants, FCDs first turned the purple colour of fresh DPPH solution into a reddish-brown colour and then gradually into a colourless (bleached) product (Fig. 6.6d). Compared to ascorbic acid, the reaction of DPPH with FCDs proceeded at a slower rate. Nevertheless, the decrease in absorbance is detectable within minutes of reaction. This observation corroborates with the DPPH radical scavenging activity of carbonaceous fullerenes (C\textsubscript{60}).\textsuperscript{43} As shown in Fig. 6.6e, the scavenging activity of ascorbic acid and FCDs increased in dose-dependent manner up to 94\% at the dose level of 90 \( \mu \)g/ml and to 89\% at the dose level of 120 \( \mu \)g/ml, respectively. The EC\textsubscript{50} value was determined from the plotted graph of scavenging activity versus the concentration of sample/standard, which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50\%. EC\textsubscript{50} values of ascorbic acid and FCDs were found to be 35.83±4.17 and 56.16±2.97 \( \mu \)g/ml, respectively (\( p<0.05 \)), confirming that the scavenging potential of FCDs was lower than that of ascorbic acid. Nonetheless, this noble facet of FCDs raises the prospects of its incorporation into oxidation-prone food models. Indeed, the nanochemistry involved in radical scavenging property of C-dots needs further investigation; however from the knowledge gained so far, it could be explained that the quenching behavior of FCDs may be attributed to its quantum size with increased surface-to-volume ratio, providing more available reaction sites, and also to the presence of different functional moieties on the surface (-OH, C=O, -CONR, -COOH, etc.), which tends to interact with and reduce DPPH species. It has been reported that among the water-soluble fullerenes, nanomaterials of \( \text{C}_{60}(\text{C(COOH)}_2)_2 \), \( \text{C}_{60}(\text{OH})_2_2 \) and metallofullerenes possess high antioxidative function and can wipe out different types of
reactive oxygen species (ROS) depending on surface modification.\textsuperscript{42} Amino acid-functionalized multi-walled carbon nanotubes were found to be more potent antioxidants than butylated hydroxyanisole and reduced glutathione.\textsuperscript{44} A schematic illustrating the probable mechanism(s) is proposed in Fig. 6.7. Data from the literature suggests that similar to fullerenes, ROS or free radicals may be “grafted” or adsorbed at the surface of C-dots by radical addition to the carbon framework, which is due to their high electron affinity.\textsuperscript{43,45} Thus, the mechanism of scavenging reaction is not straightforward in case of NPs and a generalized inference can be drawn that the organic functionalities of NPs can quench DPPH radicals (perhaps by providing hydrogen atom or by electron donation, conceivably via a free radical attack on the DPPH molecule, or by grafting/linking of radicals to C-dot’s surface) and convert them to a colourless/bleached product (i.e., 2,2-diphenyl-1-picrylhydrazine, or a substituted analogous hydrazine).

### 6.3.3. Hemocompatibility

A hemolysis test was used to determine the toxicity of FCDs on mammalian RBCs. Since there are concerns about the health implication of NPs, it is important to understand how FCDs interact specifically with RBCs, as it would eventually come in direct/indirect contact with blood as soon they are exposed to the host’s body. Hemolytic activity of NPs is usually determined by measuring the absorption peak of hemoglobin at 570 nm, which is released to the solution from hemolyzed cells.\textsuperscript{46} Photographs showing precipitated RBCs at the end of the hemolysis experiment are given in Fig. 6.8a. The red colour of the released hemoglobin from damaged cells is clearly observable for positive control and 50 mg/ml concentration of FCDs. For rest of the samples (negative control and 0.1-10 mg/ml of FCDs), the supernatants are almost colourless, confirming the intactness of the RBC membrane in these cases. This result is in conformity with that of Fig. 6.8b. According to ASTM E2524-08 standard (Standard test method for analysis of Hemolytic products of NPs), hemolysis >5% indicates that the test material causes damage to RBCs; this criterion was exceeded at the FCD concentration of 50 mg/ml. Hence, FCDs were found to exhibit hemolytic activity only at higher concentration (≥50 mg/ml), which is
much lower than that reported for their other counterparts (fullerenes, carbon nanotubes, etc.) and several similar NPs.\textsuperscript{47} This benevolent feature may be due to the presence of congenial surface functional groups, making FCDs tolerable at low concentration. Yildirim et al.\textsuperscript{46} also found that surface functionalization of nano-structures can render them completely non-hemotoxic.

To get a closer insight into the FCD-induced biological effect on RBC, the uptake of FCDs by the RBCs was examined by fluorescence microscope (Fig. 6.8c). With the increasing dosages (20, 30 and 40 mg/ml), no marked changes in the morphological shape of RBCs was detected; however, higher concentration (50 mg/ml) caused dramatic hemolysis, showing ruptured cell membrane and patches of released cell content (Fig. 6.8d). It is possible that mechanical pressure exerted by excess permeation and accumulation of FCDs inside the cells caused lysis, especially at higher dosage. Thus, FCDs were found to be biocompatible in a dose-dependent manner, with no detectable hemotoxic effect up to 40 mg/ml. The C-dots were likely internalized into the RBCs through endocytosis,\textsuperscript{16} and even after entering into the cells, the C-dots retained their fluorescence property and turned bright green under UV excitation (488 nm). In addition when the laser excitation was changed to 405 nm, a fluorescent blue color was observed. A similar observation was obtained by Sahu et al.\textsuperscript{8} in case of orange juice-derived CNP. All these preludes demonstrate that the FCDs obtained from spent-rapeseed meal can serve as a potential substitute for organic dyes or semiconductor QDs in bio-imaging application.
Fig. 6.8 (a) Photographs of RBCs treated with FCDs at different concentrations. The released hemoglobin from the damaged cells in the supernatant can be seen from the photographs; (b) Hemolysis percentages of FCDs at different concentrations; (c) RBCs treated with various concentrations of FCDs (20, 30 and 40 mg/ml) observed under fluorescence microscope by excitation at 405 nm ((i)-(iii)) and 488 nm ((iv)-(vi)). Bars with different letters indicate significant difference (p<0.05).
6.3.4. Characterization of rapeseed protein film, with (F1 and F2) or without (F0) added FCDs

Having established the biocompatibility, we introduced the FCDs into a biopolymer matrix (rapeseed protein) in a pursuit to make fluorescent bio-packaging material. One of the most important and difficult tasks in the incorporation of active compounds and new materials in a matrix is understanding if they will be miscible with the matrix and if they will not influence the processability and their structure. In this study, the preliminary experiments allowed the determination of FCD concentrations that when added to the protein-based matrix do not affect significantly their appearance and processability. Accordingly, composite films having 20 or 30 mg FCDs per 100 ml of film-forming solution were prepared for further analyses.
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

**Fig. 6.9** Photograph showing the pristine protein film and the nanocomposite films under normal light (on left) and UV light (on right) (F0 (a), F1 (b), F2 (c)); and their corresponding SEM images (F0 (d), F1 (e), F2 (f)).
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

Fig. 6.10 (a) FTIR spectra and (b) TGA curves of F0, F1 and F2.
On the basis of visual inspection, all the examined films (F0, F1 and F2) showed similar homogeneity, transparency and flexibility, with the exception of colour. The colour of the film changed from slightly yellow to dark brown-yellow, when FCDs were added (Figs. 6.9a-c). Generally, CNPs are blackish-brown, which might be the cause for dark colouration. As anticipated, F0 did not show any green glow under UV light; whereas under the same condition, F1 and F2 appeared as intense green–light emitting films. From this, it is clear that a homogeneous dispersion of the nano-component in the film-matrix is obtained, without the presence of any visible green-coloured fluorescent patches or clusters. Thus we were successful in fabricating fluorescent glowing edible films based on the luminescent C-dots, which is envisioned to be used in forgery-proof packaging. It is expected that such packaging would benefit consumers, industry stakeholders and food regulators. At this point, one of the objectives in this work was accomplished, and the comparative study of the physicochemical properties of these films was undertaken.

SEM was used to characterize the topography and morphology of the films (Figs. 6.9d-f). Microstructure of F0 presented rough ridge-like protrusions on the surface, typical of protein films, as has been also reported for films prepared with soy protein, rapeseed protein and pumpkin seed protein. Surprisingly, F1 and F2 did not show any such ridge structures; rather, a good distribution of the additive (FCDs) in the matrix was found. FCDs appeared to be embedded on the polymeric matrix and exhibited a heterogeneous size distribution most likely due to some agglomeration. Such problems of agglomeration of nanomaterials in composite films have often been reported. Agglomeration usually occurs when a relatively high loading of the nanomaterial is added to the matrix, as is the case here. Apparently such tiny clustering did not affect the green fluorescence of the films.

The effect of the addition of FCDs into protein film was initially evaluated by FTIR. When different compounds are mixed, physical bonds and chemical interactions are reflected by changes in characteristic spectral peaks. FTIR spectra of F0, F1 and F2
are shown in Fig. 6.10a. The main broad peaks are maintained, the spectra of the films with FCDs being similar to that of the control. Special mention should be made of the peaks between 3500 and 3000 cm\(^{-1}\), corresponding to the hydroxyl groups or hydrogen-bonds or to the N-H group, that are weaker in F0 when compared to F1 or F2. This stretch appeared to be more recognizable with increasing FCD concentration. This indicates higher amounts of O-H or N-H groups in F1 and F2 than in F0. Derived from this fact, it is feasible to believe that FCDs, with hydrophilic surface moieties, may form hydrogen-bonding between themselves or with amino groups of protein, especially when the protein is partially unfolded (denatured) during heating of the film-forming solution. This might be one of the possible reasons that F1 and F2 had more compact, uniform and homogeneous surface morphologies compared to F0, as viewed under SEM (Figs. 6.9d-f), mainly due to high compatibility of FCDs in protein matrix. Addition of FCDs led to the emergence of few new peaks in the region of 717-727 cm\(^{-1}\) (ascribable to out-of-plane C-H bending in aromatic substitution) and 878-881 cm\(^{-1}\) (correlating to the presence of vinyl groups). Moreover, in F1 and F2, the intensity of C-H or C-N stretch at 1400-1450 cm\(^{-1}\) increased and seemed to be more distinguishable than that in F0. All these facets signify the possibility of new interactions and cross-links between FCDs and polymeric matrix.

The thermal stability of the composite films was tested by TGA. The thermograms in Fig. 6.10b show that all of the film samples follow a similar degradation pattern, indicating uniform dispersion and high interactions of FCDs with the film (polymer) matrix.\(^{51}\) Surprisingly, in all the 3 samples, no significant mass loss was noted up to \(\approx\)260 °C, beyond which decomposition of glycerol and protein occurred.\(^ {18}\) The char yield is the non-volatile carbonaceous material generated on pyrolysis, which is indicated by the residual weight after the decomposition step. It is worth mentioning that the increase of the residual yield (the char) with the increase of FCD concentration was detected, which points to the thermo-insulating nature of the C-dots as a nanofiller for the polymer matrix, a property similar to that of carbon nanotubes.\(^{52}\) After degradation, the net weight loss of F0 was found to be \(\approx\)80.58%; whereas those of F1 and F2 were
≈59.73% and ≈45.65%, respectively. Thus, with the increase in FCD content, significant (p<0.05) reduction in weight loss was observed, thereby proving FCDs as potent thermo-insulators for proteinaceous films, a property desirous for high temperature processing.

Although the same amount of film-forming solution was used in all the three films, the slightly higher thickness of F0 (Table 6.5) may be due to its ridges-like rough outgrowths on its surface, as seen under SEM (Figs. 6.9d-f). Presence of FCDs led to an increase of water affinity of edible films, showing that moisture content increases in F1 and F2 significantly (p<0.05) as compared to F0 (Table 6.5). This may be due to the hydrophilic behavior of the –OH groups in FCDs, which probably influenced WVP, increasing the adsorption of water molecules in FCD-incorporated films when compared with F0. As a consequence, the water vapour barrier property of F1 and F2 is lesser than F0 (Table 6.5). The opposite behavior was observed for O₂ permeability, leading to a significant decrease (p<0.05) of the values for FCD-containing films when these were compared with F0. Usually it is perceived that higher WVP would render higher O₂ permeability; however in this case, the radical scavenging functional groups of FCDs probably created additional sites in the film favouring the removal of oxygen, thereby minimizing the influence of adsorbed moisture in the film’s transport properties. Thus the oxygen barrier property is improved due to the presence of FCDs.

TS, puncture strength and EB of a material are used to study their resistance to tensile stress, to determine the breaking force and to express the percentage of increase in length that occurs before the sample breaks (i.e. film’s ability to stretch), respectively. The presence of FCDs in F1 and F2 influences their mechanical properties, decreasing the values of TS and puncture strength (p<0.05); on the other hand, an increase of EB values was observed (Table 6.5). The reason behind the decrease in TS and puncture strength by the addition of FCDs was the hydrophilic nature of C-dots which favours the interaction with water molecules, as explained by the higher moisture content of the films. Due to their small sizes, FCDs fit easily into the polymer chains. Because of the hydrophilic groups of FCDs (especially –OH), it is likely to be strongly bonded by
hydrogen bridges with glycerol and protein molecules especially at amine, amide, carboxyl and hydroxyl sites.\textsuperscript{53} As a result, protein-protein interaction decreases and polymer segmental mobility increases (flexibility or EB enhances).

### Table 6.5 Physico-chemical properties of pristine protein (F0) and nanocomposite film (F1 and F2)\textsuperscript{§}

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<th>Property</th>
<th>F0</th>
<th>F1</th>
<th>F2</th>
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<tr>
<td>Thickness (μm)</td>
<td>54.1±0.2\textsuperscript{a}</td>
<td>48.8±0.15\textsuperscript{b}</td>
<td>49.7±0.09\textsuperscript{b}</td>
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<td>Moisture content (% dry basis)</td>
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<td>35.8±1.1\textsuperscript{b}</td>
<td>37.9±1.4\textsuperscript{c}</td>
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<td>WVP (ng m/m\textsuperscript{2} s Pa)</td>
<td>1.4±0.62\textsuperscript{a}</td>
<td>2.5±1.6\textsuperscript{b}</td>
<td>2.9±1.4\textsuperscript{b}</td>
</tr>
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<td>Oxygen permeability (ng d\textsuperscript{-1} Pa\textsuperscript{-1} m\textsuperscript{-1})</td>
<td>8.6±0.30\textsuperscript{a}</td>
<td>4.7±0.2\textsuperscript{b}</td>
<td>2.9±0.20\textsuperscript{c}</td>
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<td>Puncture strength (N)</td>
<td>1.2±0.03\textsuperscript{a}</td>
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<td>Elongation at break, EB (%)</td>
<td>9.6±0.02\textsuperscript{a}</td>
<td>17.8±0.69\textsuperscript{b}</td>
<td>16.1±1.03\textsuperscript{b}</td>
</tr>
<tr>
<td>Hunter-L</td>
<td>81.0±0.13\textsuperscript{a}</td>
<td>78.2±0.16\textsuperscript{b}</td>
<td>77.2±0.09\textsuperscript{b}</td>
</tr>
<tr>
<td>Hunter-a</td>
<td>0.34±0.01\textsuperscript{a}</td>
<td>1.2±0.03\textsuperscript{b}</td>
<td>1.6±0.05\textsuperscript{c}</td>
</tr>
<tr>
<td>Hunter-b</td>
<td>11.9±0.12\textsuperscript{a}</td>
<td>10.9±0.07\textsuperscript{b}</td>
<td>8.6±0.04\textsuperscript{c}</td>
</tr>
<tr>
<td>Opacity</td>
<td>2.4±0.17\textsuperscript{a}</td>
<td>2.5±0.13\textsuperscript{b}</td>
<td>2.6±0.02\textsuperscript{b}</td>
</tr>
<tr>
<td>DPPH scavenging activity (%)</td>
<td>3.7±1.6\textsuperscript{a}</td>
<td>64.9±2.8\textsuperscript{b}</td>
<td>71.7±4.0\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{§}Results are mean±standard deviation of 3 replicates. Values followed by same superscript letter within a row are not significantly different (p>0.05)

Good optical properties of film are extremely important in food packaging for ensuring that the consumers can clearly see the food product. The darkness of the FCD-loaded films was significantly higher (p<0.05), as evidenced by the lower Hunter-L values compared to the control. Meanwhile, in comparison with F0, increase in FCD concentration increased Hunter-a (indicator of the tendency towards redness) and decreased Hunter-b (indicator of the reduction of yellowness) values. F0 was more transparent (lower opacity value) than F1 and F2 (Table 6.5). The opacity of the film samples significantly increased (p<0.05) with increasing FCD concentration. This
observation could be explained by the fact that a higher amount of added FCDs hinders light passage through the film. This result accords with several published studies in which addition of antioxidants, polyphenols, plant extracts or other additives to film matrices, has been found to affect the optical properties severely.

As the level of FCDs increased in the film formulation, so did the expected antioxidant character of the composite film (Table 6.5). In relation to F0, the DPPH scavenging activity increased by 17.5 and 19.3 fold in F1 and F2, respectively. F0 also showed some scavenging activity on DPPH. This is related to the fact that free radicals can react with the residual free amino (-NH$_2$) groups to form stable macromolecular radicals, and the -NH$_2$ groups can form ammonium (NH$_3^+$) groups by absorbing a hydrogen ion from the solution.$^{25}$ It should be noted that although a high amount of FCDs was incorporated into the film, the FCDs in the protein film showed much lower antioxidant capacity than in the free form (aqueous dispersion) at much lower concentration (Fig. 6.6e). This could be due to interaction between FCDs and the film matrix formed via hydrogen bonds and other cross-links, thereby reducing the availability of free structural factors such as hydroxyl, keto, carboxylic groups, etc.,$^{54}$ which imparts a scavenging feature to FCDs.

### 6.3.5. Oxidative stability analyses of rapeseed oil packed in nanocomposite films

On the basis of the above results, it was presumed that the use of FCD-incorporated composite packaging could be helpful in improving the oxidative shelf-life of lipid food items during storage. As such, rapeseed oil, packed in small pouches made of either nanocomposite films (F1 and F2) or pristine protein film (F0), was monitored over time to assess the antioxidative effect of these films on a real food system. The samples packaged in nanocomposite films (S1 and S2) can easily be segregated from the control (S0) under UV light, on the basis of the inherent green luminescence of C-dots (Fig. 6.11a). To consider the complexity of the lipid oxidation process, both the primary and secondary oxidation products have been assessed.
Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

**Fig. 6.11** (a) Oil sample packed in F0, F1 and F2, as viewed under normal light and UV light; Changes in (b) Free fatty acids (FFA); (c) Peroxide value (PV); (d) Conjugated dienes (CD); and (e) TBARS values of S0, S1 and S2 during storage.
Within the first 14 days of storage, FFA content increased perceptibly in both S0 and S1 (p<0.05); however, S2 showed negligible changes as compared to day 0 (p>0.05) (Fig. 6.11b). Subsequently, a gradual increase was found up to 21 days, and the highest FFA value was obtained on day 28. Possibly, lipid hydrolysis, catalyzed by lipases, occurred to a greater extent at the end of the storage period. On day 28, the respective FFA values of S1 and S2 were ≈1.2 and ≈1.4 fold less than that of S0. A small amount of FFA is usually noticeable in cold-pressed oil due to the release of enzymes from crushed seeds along with the oil. Alternatively, the accumulation of FFA could be attributable to the lipases from micro-organisms, which probably enhanced with extended storage.\(^{32}\)

Unsaturated fatty acids react with molecular oxygen, usually via a free radical mechanism, to form hydroperoxides, the primary oxidation products.\(^{55}\) A marked increase in PV was observed in all the tested samples for up to 14 days (p<0.05) (Fig. 6.11c). Thereafter, a decrease in PV was noticeable with extended storage (p<0.05), which was presumed to be due to degradation of hydroperoxides, yielding a wide variety of secondary decomposition products including aldehydes. On day 28, the PV of S0 was almost 2 fold higher than those of S1 and S2, which indicates the higher deterioration rate of S0 compared to the other samples.

During oxidation of PUFAs containing methylene-substituted dienes and polyenes, there is a shift in the position of the double bond due to isomerization and conjugate bond formation (conjugated dienes).\(^{33}\) This is accompanied by increased UV absorption at 233 nm. It is an indicator of auto-oxidation and is reported to increase with uptake of oxygen and formation of peroxides, during the early stages of oxidation. Chaijan et al.\(^{32}\) reported that almost immediately after peroxides are formed, the non-conjugated double bonds (C=C-C=C) that are present in natural unsaturated lipids are converted to conjugated double bonds (C=C-C=C). Thus, conjugated dienes are the primary oxidation products formed. \(A_{233}\) of S0 increased slightly up to 14 days of storage, after which its value showed a decreasing trend (Fig. 6.11d). S1 and S2 exhibited similar profiles up to 21 days. From day 21 onwards, \(A_{233}\) of S1 decreased and that of S2
continued to increase, till the end of storage period (p<0.05). This decrease probably occurred because of their decomposition into secondary oxidation products.

Secondary lipid oxidation was studied by the TBARS value. Traditionally, the absorbance at 532 nm of the pink pigment formed in the reaction is measured. In all of the samples, TBARS value increased sharply throughout the storage (p<0.05) (Fig. 6.11e). The marked increase in TBARS during 14-28 days of storage was coincidental with the decrease in PV (Fig. 6.11c). This was probably due to the destruction of hydroperoxides into relatively polar secondary products, especially aldehydes in the later stages of lipid oxidation. It was most likely that a higher rate of lipid oxidation might be taking place at the end of storage period (days 14-28). Additionally, the loss in natural antioxidants (such as tocopherol, polyphenols, etc.) of rapeseed oil during extended storage might contribute to the increased lipid oxidation.

On the basis of the available data, it can be interpreted that the antioxidative FCDs in F1 and F2 retarded the lipid oxidation rate in S1 and S2 by quenching the free radicals. The formation of free radicals, which precedes lipid hydroperoxides, is the initial step in lipid oxidation. Abstraction of hydrogen atoms from allylic carbon atoms in unsaturated lipids generates a lipid radical, which in-turn has the potential to initiate the chain reactions of the auto-oxidation cycle. The cycle is terminated either through a recombination of two radicals or by a reaction of the radical with antioxidant, leading to the formation of more stable radicals. The chain-breaking antioxidant, therefore, interrupts the oxidation chain reaction to enhance stability. Thus, FCD-incorporated packaging materials showed a discernible impact on the oxidative shelf-life of lipid product. Further studies need to be undertaken to improve the antioxidative and antimicrobial properties of these composite films.
6.4. Conclusion

Arising from this work, it is concluded that high-value CNPs can be easily synthesized from spent rapeseed meal by a simple green hydrothermal route. The obtained NPs were found to be multi-functional such as water solubility, high reductive potential, photoluminescence and biocompatibility. This is due to the rich functionalities attached to the nano-carbons. Literature enumerates biopolymer-based films as good vehicles for the incorporation of active compounds; however, inclusion of fluorescent carbon nanostructures has rarely been evaluated. The present investigation shows the incorporation of FCDs into rapeseed protein film matrix, which adds new features to the film; for instance photoluminescence, enhancement in antioxidant potential and thermal stability. Moreover, oil samples packed in sachets made of FCD-protein composite film, are able to resist oxidation better than that stored in pristine protein-based sachet. The work opens up new possibilities for agricultural residues as a valuable precursor of useful nanomaterials, and can subsequently give rise to a new concept of bio-based edible fluorescent food packaging, suggesting new scalable and simple approaches to improve environmental sustainability in industrial processes. Further studies should be undertaken to analyze the release behavior of FCDs from the film into different food model systems, their effect on organoleptic properties, gastro-intestinal tract, and toxicological assessment using in-vitro and in-vivo models.

6.5. References


Chapter 6: Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film


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