Hybrid Bionanocomposites from Spent Hen Proteins
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Supporting Information

ABSTRACT: Spent hens, a poultry by-product, have little economic value for processing and mostly end up in landfills. However, there are concerns over disposal of spent hens; therefore, it is pertinent to find out alternative uses that are environmentally sound. On the other hand, single-use plastic packaging is leading to a global environmental crisis. In this study, proteins were extracted from spent hen, plasticized, and processed into films by compression molding. The hybrid bionanocomposite films were successfully prepared using glycerol as a plasticizer, chitosan as a cross-linker, and varying concentrations of nanoclay as a nanoreinforcement. The effects of nanoreinforcements, plasticization, and cross-linking were then evaluated on thermal, mechanical, and barrier properties of the prepared bionanocomposite films. Various concentrations of nanoclay and chitosan were dispersed in the protein matrix. However, with the same plasticizer loading, the optimum addition of chitosan and nanoclay led to almost twofold increase in the mechanical strength, compared to neat protein films. The results indicated that at optimal conditions, a good intercalation and/or exfoliation of the protein biopolymers into clay interlayer galleries was observed leading to improved thermal, thermomechanical, and barrier properties. These hybrid bionanocomposite films have great future potential to be used in packaging and other applications.

1. INTRODUCTION
Currently, global annual production of plastics is over 300 million tons with an annual growth rate of about 5%. Crude oil resources are the main source of plastic production.1 Packaging is the third largest plastic application sector around the globe and represents 2% of GDP in the developed world. Worldwide, there was 31% increase in total municipal waste from 1988–2005, and packaging was a major (37%) contributor.2 Presently, the plastic market is growing rapidly with an annual growth rate of 30%.3 Food packaging is the most significant application of packaging, contributing almost 2/3 of a total packaging waste.4 In the present linear plastic economy, 90% of products is single-used and discarded, leading to a major source of plastic litter.5 The extensive use of nondegradable synthetic plastics for packaging is leading to concerns for the environmental security and sustainability. The excessive reliance on petroleum-based packaging materials can be eased by bioplastic expansion using sustainable and renewable resources.6 Bioplastics represent a wide spectrum of thermoplastic materials which are obtained from biological resources or combination of both biological and fossil fuel resources. Many studies are currently being carried out to make new monomers from biological resources either by industrial biotechnology or by chemical processes. Recently, various reports have been published on utilization of waste proteins for applications in packaging, aerospace, sports, automotive, and construction industry.5−10 Kaur et al. 2018 synthesized nanoreinforced green bionanomaterials from chicken feather keratin proteins and suggested for multiple commercial applications.10 Another study is reported by de Oliveira and co-workers in 2019 prepared biopolymer films from a by-product of cheese industry. That is, whey protein isolates as a smart packaging prepared biopolymer films have great future potential to be used in packaging and other applications.

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functions such as antimicrobial, biosensing, and oxygen scavenging properties.\textsuperscript{14,19,20} For the production of bio-based plastic materials, the use of biopolymers from industrial by-products is highly desirable because it not only reduces utilization of food grade protein biopolymers such as wheat and corn but also diverts waste from landfills. One such potential source of proteins is spent laying hens which are mostly disposed. Because of low yield and poor quality, the meat products made from spent hen are not able to compete with broiler meat and therefore no longer economically viable. The current disposal methods for spent hen such as burying, composting, and incineration are often both environmentally and economically unfavorable. Therefore, alternatives uses of spent hens are needed to be explored.\textsuperscript{21,22}

In Canada, approximately 26 million egg-laying hens are produced yearly.\textsuperscript{23} Each spent hen has \textasciitilde{}1.8 kg meat, and on average, the whole carcass contains about 17% proteins, which represents about 8–10 kilotons of proteins only in Canada. Herein, we report an integrated approach to harvest proteins from whole spent birds and develop bionanocomposite materials using nanoclay as reinforcement. Nanoclays are considered one of the most affordable nanofillers and are well known to enhance properties of polymeric materials if dispersed effectively. The properties of the developed bionanocomposite materials were investigated in detail. To the best of our knowledge, no report on the development of bionanocomposite packaging films from spent hen proteins is available in the literature.

2. RESULTS AND DISCUSSION

2.1. Proteins’ Purity and Recovery. The high recovery of proteins is not only important from an economic stand point but also to determine the efficiency of the extraction method. The protein recovery achieved by alkali-aided method mainly depends on three factors: the solubility of protein at a high pH, sediments size after centrifugation, and the solubility at the isoelectric point.\textsuperscript{24} The alkali-aided method has been used for fish muscles, chicken residue, and mechanically separated turkey meat. However, in this study, we used this method with slight modification for the whole spent hen to extract the proteins. Higher alkaline pH was considered for the current study. Studies have shown that protein’s solubility at higher pH has been attributed to increased net negative charges on protein chains, leading to electrostatic repulsion between the chains. At isoelectric point, protein molecule carries no net electrical charge; therefore, charged groups are least available for interactions with water molecules, and further exposure of nonpolar/hydrophobic amino acids may cause increased precipitation because of thermodynamically unfavorable interaction between water and nonpolar groups ultimately leading to higher protein recovery.\textsuperscript{25} The same phenomenon is observed here.

The high protein recovery was contributed by the increased solubilization at higher pH (12) and later precipitation at 5.75 pH. The protein recovery yield and purity with our method was 74 and 96%, respectively, which is quite higher in comparison to protein extraction available in the literature. Omana et al.\textsuperscript{26} reported the protein recovery yield of \textasciitilde{}66% with final protein contents of 19.6% from turkey meat. Recently, functional proteins were extracted from the bovine and porcine hearts using acid or alkali solubilization and isoelectric precipitation. They reported the protein recovery between 51.53 and 55.74%.\textsuperscript{27}

2.2. Mechanical Strength. Stress–strain curves are commonly used to study the mechanical properties of polymers. The tensile strength and elongation at break are typically examined properties to determine the polymer applications. The materials having tensile strength lower than 5 MPa and elongation more than 100% are considered as a rubbery material. On the other hand, glassy materials have tensile strength more than 30 MPa and no elongation.\textsuperscript{28} Commonly, thermoplastic materials which are used for packaging purposes having tensile strength up to 10 MPa and elongation at break around 1000% such as low density polyethylene\textsuperscript{29} and polybutylene succinate\textsuperscript{30} derived packaging materials. The neat protein films without plasticizer were too brittle to perform tensile tests. Therefore, a number of plasticizers were used to achieve the required thermoplastic behavior of the proteins derived films. Glycerol was found to be the most suitable and compatible plasticizer among sorbitol, ethylene glycol, poly(ethylene)glycol, and butanediol.

The nanoclay concentrations (1, 3, 5, and 8%) were used to find out its optimum percentage to obtain maximum mechanical strength. The films containing 5% nanoclay displayed better mechanical properties compared to the films prepared with other concentrations of nanoclay (1, 3, and 8%). The mechanical properties of these films are given in the Supporting Information (Table S1). The films with better tensile strength were considered for further characterization, and understanding the effect of the clay on spent hen derived proteins material. The blends were denoted as PG1 (proteins with 20% glycerol), PG2 (proteins with 25% glycerol), PGC1 (proteins with 20% glycerol and 3% chitosan), PGCB1 (proteins with 20% glycerol, 3% chitosan, and 5% nanoclay), PGCB2 (proteins with 25% glycerol and 3% chitosan), and PGCB2 (proteins with 25% glycerol, 3% chitosan, and 5% nanoclay). In all above-mentioned blends, moisture was kept constant which was 10%.

The stress–strain curves of the resulting films are presented in Figure 1, and values are summarized in Table 1. Table 1 shows the stress–strain curves of films with glycerol, glycerol/chitosan, and glycerol/chitosan/nanoclay. The results revealed an increment in mechanical properties by the addition of 3% [Figure 1. Stress–strain curves of PGC1, PGCB1, PGC2, PGCB2, PG1, and PG2.]

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Table 1. Stress and Strain of the Films

| bionanocomposite film | glycerol (%) | chitosan (%) | nanoclay (%) | stress (MPa) | elongation at break (%) |
|----------------------|--------------|--------------|--------------|-------------|------------------------|
| PG1                  | 20           |              |              | 2.43 ± 0.50 | 474 ± 10.20            |
| PG2                  | 25           |              |              | 2.65 ± 0.60 | 474 ± 11.30            |
| PGC1                 | 20           | 3            |              | 5.81 ± 1.20 | 484 ± 06.70            |
| PGCB1                | 20           | 3            | 5           | 11.37 ± 2.45| 322 ± 05.60            |
| PGC2                 | 25           | 3            |              | 4.57 ± 1.35 | 1590 ± 20.60           |
| PGCB2                | 25           | 3            | 5           | 7.33 ± 2.50 | 853 ± 15.40            |

*Reported values are mean of three replicates ± standard deviation. Means in the same column followed by the same superscript letter is not significantly different (P > 0.05).*

chitosan. Further, by addition of 5% nanoclay, a remarkable enhancement in mechanical strength was observed (11.37 MPa). From Table 1, it can be clearly seen that films prepared using 25% glycerol with 3% chitosan have maximum elongation, that is, 1590%. The plasticizer usually increases using 25% glycerol with 3% chitosan have maximum elongation, that is, 1590%. The plasticizer usually increases the flexibility and extensibility of the material. Further the addition of chitosan improved the mechanical strength of the films probably because of ionic interactions between chitosan and protein chains, chitosan’s film-forming ability, and H-bond network formation in the solid state. 31,32

Furthermore, by the addition of nanoclay, the tensile strength of the films was improved and extension decreased. This could be due to hydrogen bonding, electrostatic interactions between the nanoclay, protein, and chitosan, leading to improvement in the mechanical strength of protein/chitosan/nanoclay hybrid films. 3. As evident, the maximum improvement in tensile strength is at the addition level of 5% clay most likely because of the enhanced interactions’ among components of films as described above and still relatively homogeneous dispersion of nanoparticles compared to higher concentration of nanoclay as confirmed from the transmission electron microscopy (TEM) images. The well-dispersed nanoparticles enhance the strength as reported by other researchers in the case of layered particles. 34

2.3. Structural Analysis. Fourier transform infrared attenuated total reflection (FTIR ATR) was used to understand the structure of pure proteins, clay, and prepared films, as shown in Figure 2a,b. All of the films and pure proteins show characteristic absorption bands of polypeptide as amide A, amide I, amide II, and amide III. 3. The absorption band at 3277 cm⁻¹ is ascribed to the stretching vibrations of O–H and N–H (amide A), whereas the band at 2932 cm⁻¹ is related to the symmetrical C–H stretching vibrations. 3 The strong absorption band at 1633 cm⁻¹ is assigned to the C=O stretching with minor contribution from C–N stretching (amide I), providing information related to proteins’ secondary structure.

The amide II region is related to the 1538 cm⁻¹ absorption band because of N–H bending and C–H stretches. A weak band at 1234 cm⁻¹ is assigned to the amide III, which is the combination of C–N stretching and N–H in plane bending along with the influence from C–C stretching and C=O bending vibration. 39 These three bands give evidence about the protein conformation and backbone structure. Literature suggested that the peak at 3277 cm⁻¹ (amide I) is due to the α-helix structure; the range of 1539–1515 cm⁻¹ (amide II) is attributed to the β-sheet structure. 30,40 Therefore, all of the derived films possess two microstructures of α-helix and β-sheet.

From Figure 2a, a clear change can be seen in intensity and position of absorption signals around 3200, 2950, 1627, and 1525 cm⁻¹ in bionanocomposites as compared to pure proteins. The shift in peak position with change in intensities shows the interaction between proteins and clay. Particularly, there is a significant increase in intensity around 3200 cm⁻¹, assigned to OH stretching vibrations, which may be attributed to the presence of more hydrogen bonds between layers of clay and proteins in composites. The band around 1000 cm⁻¹ can be assigned to Si–O–Si asymmetric stretching vibrations of nanoclay, whereas silanol hydroxyl group shows band at 3605 cm⁻¹ in the FTIR spectrum of neat clay. 12 In the FTIR spectrum of bionanocomposites in Figure 2a, the band around 1000 cm⁻¹ is shifted to 1031 cm⁻¹, whereas in the hydroxyl region, a broad band is obtained. These changes in both regions revealed the interaction between protein matrix and nanoclay.

Figure 3 shows the amide II region spectra of pure proteins and derived composites. This region is much more sensitive to the environment of N–H group. Therefore, this region is very
useful to track changes to the environment of N–H group and responds differently in hydrogen-bonding environment. Generally, N–H groups which are strongly hydrogen bonded absorb at higher frequencies. There is a clear increase in relative intensity around 1545 cm\(^{-1}\) in all derived films as compare to the pure proteins which is related to the presence of stronger hydrogen-bonded N–H group in the composites. The clay present in the bionanocomposites can form a greater number of hydrogen bonds; as a result, number of hydrogen-bonded peptide groups is increased.

Figure 4 represents the second derivative region (1615–1680 cm\(^{-1}\)) of all films along with pure proteins. It allows the direct separation of amide I band into its components and is used to identify the position of individual amide I bands. Second derivative analysis revealed the absorption band as negative bands in the second derivative spectrum. The main component bands exhibited at 1651, 1658, and 1666 cm\(^{-1}\) in films can be assigned to \(\alpha\)-helices and \(3_{10}\)-helices respectively. The peak at 1674 cm\(^{-1}\) can be assigned to antiparallel \(\beta\)-sheets/aggregated strands. A significant difference can be observed in the case of PGCB1; a band at 1638 cm\(^{-1}\) may be due to the fact that glycerol with clay promotes the formation of higher number of \(\alpha\)-helix and makes more interaction with the proteins.

2.4. Crystallinity Study of Bionanocomposite Films. X-ray diffraction (XRD) analysis is an important method to determine the crystal structure and evaluate changes in the crystallinity patterns of protein biopolymers and nanoclays. Figure 5a,b shows the XRD patterns of pure nanoclay, pure proteins, and bionanocomposites. The bentonite showed characteristics diffraction peaks at 2\(\theta\) angle of 6.13°, 19.83°, 28.58°, and 35.04°, whereas pure proteins exhibited diffraction peaks around 9°, 19.28°, 37.60°, and 43.80°. The peak at about 2\(\theta\) of 9° is commonly assigned to both \(\alpha\)-helix and \(\beta\)-sheet structures. By comparing the XRD pattern of composite samples with pure proteins, all composites showed broad peak at about 19° which corresponds to the \(\beta\)-sheet structure, whereas the peak at 17.8° relates to the diffraction pattern of \(\alpha\)-helix. These two peaks cannot be clearly assigned because of overlapping signals and resulting in a broad single peak around 19.30°. In this perspective, all of the derived composite films contain both \(\alpha\)-helix and \(\beta\)-sheets which is consistent with the results from FTIR, whereas all other characteristics peaks of pure proteins and clay are either completely disappeared or greatly reduced in the bionanocomposites which showed that proteins had developed interactions with the plasticizer and clay. The differences are more prominent in clay-based bionanocomposites which can be seen in XRD diffractogram of PGCB1 with the appearance of new distinct
crystallinity peaks. These variations might be associated with the crystal lattice volume and constant that can alter the crystal plane; thus, the polymer can be intercalated or exfoliated into the clay nanoplatelets, and the new crystal plane was formed during the process.\textsuperscript{48,49}

The peaks assigned to nanoclay at $2\theta$ of 6.13°, 19.83°, 28.87°, and 35.04° completely disappeared in the case of PGCB1. In addition to that, three new distinct crystallinity peaks can be observed in PGCB1 at $2\theta$ of 27.36°, 31.39°, and 45.41°, showing additional crystallinity regions as compared to other nanoclay-derived film (PGCB2) probably due to some new rearrangements leading to crystallizations. However, in the case of PGCB2, the peak around 9° disappeared and intensity of peaks at 37.60° and 43.80° is greatly reduced as compared to pure proteins.

As proteins are semi-crystalline polymers, conformation of chains and resulting crystallinity is prone to surface interactions. Nanofiller has a tendency to alter the degree and rate of crystallinity with better mechanical properties particularly strength because of the reinforcing effect promoted by the enhanced interactions between biopolymers and nanoparticles and development of some new regions of crystallinity.\textsuperscript{50} The similar phenomenon is observed in nanoclay. The native crystallinity is disrupted and new crystallinity regions are generated leading to improved mechanical strength as compare to PGCB2. The studies have also shown that materials’ physical properties particularly tensile strength depend on the molecular order.\textsuperscript{53} This might be the reason of highest tensile strength of PGCB1 among other bionanocomposites that is consistent with the mechanical strength data given in Table 1 and FTIR second derivative which showed more interactions between protein and nanoclay.

2.5. Effects on Thermal Properties. Thermal properties of the bionanocomposite films were studied using differential scanning calorimetry (DSC) by heating each sample from 25 to 300 °C as represented in Figure 6. The first peak ranging from 116.05 to 162.83 °C in all bionanocomposite films is related to the loss of bound moisture present in the protein matrix.\textsuperscript{51} The water loss peak for bionanocomposites containing 20% glycerol, that is, PGC1 and PGCB1, was at higher temperatures 139.0 and 162.83 °C, respectively, compared to bionanocomposites with 25% glycerol (PGC2 and PGCB2). This might be attributed to the greater availability of loosely bound moisture in the case of 25% glycerol. In the case of PGCB1, a water loss peak at higher temperature as compared to other bionanocomposite attributed to better clay interactions with the polar groups of proteins leading to decreased number of polar groups available for moisture uptake through hydrogen bonding with water molecules. Furthermore, the bound moisture present at the interior of bionanocomposites is surrounded by nanoclays which make loss of this moisture difficult; therefore, a delayed peak is observed in the case of PGCB1. The pure proteins have a peak at 209 °C might be the glass transition ($T_g$) of the proteins\textsuperscript{52} but the same peak is delayed in all bionanocomposites falling around between 235 and 250 °C. Further, the incorporation of nanofiller has an effect on the amorphous region of the proteins matrix and might cause the shift in $T_g$ of bionanocomposites toward higher temperature. The change in $T_g$ demonstrated higher thermoplasticity of derived films as compare to pure proteins. Also, melting ($T_m$) of proteins do not have big difference because of the shift in $T_g$. The melting peak ($T_m$) of proteins is around 282 °C, and same peak in PGC1 and PGCB2 is at around 260 and 273 °C, respectively. The melting region is not obvious in PGCB1 and PGCB2, might be addition of clay increased their thermal stability due to greater interaction of plasticized proteins with the clay which could be ascribed to the presence of protein biopolymer into clay galleries.\textsuperscript{55} In addition, some of the hydrophilic groups of clays interact with hydrophilic groups of proteins, which was originally present in the interior of the protein film. This is consistent with tensile strength values as they have greater strength as compare to PGC1 and PGC2.

2.6. Thermal Stability. The thermogravimetry and differential thermogravimetry (DTG) curves of pure proteins and derived films are presented in Figure 7. The weight loss in all films was observed 3–4% before 100 °C which is due to the release of free water.\textsuperscript{54} Up to 188 °C, all four bionanocomposites are thermally stable, followed by a loss which occurs due to degradation/evaporation of glycerol (glycerol rich region). The sharp weight loss was observed between 250 and 405 °C, rose up to 70% which is related to the proteins’ helix structure, destruction of peptide chain/bridge linkages.\textsuperscript{56} At 75 °C, the weight loss in bionanocomposites went up to 84%, attributed to the protein skeleton decomposition and conversion into lighter elements.

The weight loss in the case of PGCB1 is the least and remaining solid residue was 23% which was highest among all derived films. The addition of nanoclay can delay the degradation temperature and form inorganic network that act as a barrier against the gas transfer. Thus, the diffusion of pyrolysis gases is hindered.\textsuperscript{55} In other words, the thermal stability was improved with the intercalated/exfoliated structure, which was due to the movement of the biopolymer chains being reduced by the incorporation of nanoclay.\textsuperscript{57} The dispersion of clay within the protein matrix may reduce the nature or degree of interactions (hydrogen bonding, van der waals or ionic) between protein polypeptide chains which hold them together, and thus properties of the composites are altered. Similarly, better dispersion of nanoclay in the protein matrix in PGCB1 disturbed the interactions to a greater degree between protein chains as evident from the FTIR, XRD, and

![Figure 6. DSC heat flow signals of pure proteins (P), PGC1, PGCB1, PGC2, and PGCB2.](https://doi.org/10.1021/acsomega.8b03501)
DSC. Further, the result is very well supported from the tensile strength of PGBC1, that is, highest among all derived films.

In the DTG curve, the minimum of DTG curve depicts the maximum weight loss at that specific temperature. The DTG analysis curve represents two main degradation stages, first loss was up to 24% between a temperature range of 185 to 194°C which can be ascribed to loss of plasticizer (glycerol) present in the glycerol-rich zone. From DTG graph, it clearly represents for all of the blends, the weight loss starts from around 230°C and reaches a maximum decomposition (24−46%) between 310 and 326°C that is related to the protein-rich zone where glycerol is bonded to proteins strongly.

2.7. TEM Analysis. Bionanocomposite films were also analyzed by TEM to observe nanoclay incorporation and its dispersion/aggregation within the protein polymer matrix. TEM images shown in Figure 8 clearly represent the presence of dark lines of nanoclay sheets in PGCB1 (Figure 8B) and PGCB2 (Figure 8C) bionanocomposites, whereas the TEM image of neat sample PGC1 (Figure 8A) has no such dark lines which was taken as a control. TEM images of both bionanocomposites (PGCB1 and PGCB2) displayed exfoliation as well as intercalation of nanoclay sheets in the polymer matrix as have been marked in Figure 8B,C.

In PGCB2 revealed relatively less dispersion of nanoclay in the matrix with the intercalated region and the presence of some stacked layers of nanoclay (Figure 8C), whereas PGCB1 exhibited clear intercalation and exfoliation of nanoclay as marked in Figure 8B. Dispersion as stacks of silicate layers are uniformly dispersed in the polymer. This distribution can be attributed to the insertion of protein chains into the galleries of nanoclay which results to good exfoliation/intercalation of silicate layers. The homogeneous dispersion in the polymer matrix is well supported and confirmed by the XRD investigation, where new crystallinity peaks were appeared and nanoclay crystallinity peaks have been greatly reduced in the PGCB1 bionanocomposite.

2.8. Viscoelastic Properties. Dynamic mechanical analysis (DMA) is a technique, used to measure the viscoelastic properties of the polymers with changing temperature. Proteins experience several thermal transitions on heating or cooling as they are semi-crystalline polymers. Thermal transitions are generally related to the chain mobility of the polymers56 and determined from the peak of the tan δ curve.

Generally, DMA analysis is represented as a function of temperature with storage modulus and damping or tan δ. Two transitions in both the storage modulus and tan δ values of all bionanocomposites are seen in Figure 9a,b. These may be attributed to glycerol-rich and protein-rich domains.57 Literature suggested that glycerol promotes the formation of disordered structure and leads to two glass transitions, assigned to glycerol-rich and protein-rich domains.57,58

Figure 9a clearly represents that at −90°C, PGCB1 has the highest E′ as compared to all other bionanocomposites. With the increase in temperature, a rapid decline in E′ was observed for PGC1 and PGC2 bionanocomposite films, whereas clay-based bionanocomposites showed a gradual decrease. The decrease was more prominent from 74 to 144°C in PGC1 and PGC2, whereas PGCB1 and PGCB2 exhibited higher and steady decrease in E′. Studies have shown that higher storage modulus is observed because of the more interactions between filler and matrix.8

Figure 9a shows that the drop in E′ value of all blends is like synthetic polymers and such relaxation is thermoplastic material-related characteristic. The transitions for all blends represent a good compatibility of glycerol with proteins.31 The
low molecular weight of glycerol enables it to incorporate itself into the polymer chain and lubricate it easily. Thus, the protein—glycerol interaction is developed at the expense of protein—protein interaction. This is in agreement with the free volume theory of the plasticization. The storage modulus value of film PGBC1 is highest among all films. This is in agreement with the FTIR data, as higher value of E’ means higher volume fraction and better interaction between matrix and nanoparticle.

Two transitions in the tan δ graph of all bionanocomposites are shown in Figure 9b. These may be attributed to glycerol-rich and protein-rich regions. Similar transitions have been found in the literature for other glycerol plasticized films which can be assigned to glycerol-rich and protein-rich domains. The storage modulus value of film PGBC1 is highest among all films. This is in agreement with the FTIR data, as higher value of E’ means higher volume fraction and better interaction between matrix and nanoparticle.

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glycol, polyethylene glycol, nanoclay (bentonite), HCl, and NaOH were purchased from Sigma-Aldrich and used as received.

4.2. Protein Extraction. For the extraction of proteins, McCreedy method was used with some modifications.66 The whole spent hen sample was mixed with ice cold distilled water (1:4) by using a homogenizer for 15 min. The pH of the resulting mixture was raised to 12 by using 2.0 M NaOH under constant stirring. Further, the slurry was centrifuged (13 000g) at 4 °C for 10 min using a controlled temperature centrifuge. Thus, three layers were formed, an upper layer of fat, middle layer of soluble proteins, and a bottom insoluble layer. The middle layer, containing proteins, was separated carefully, and pH was adjusted to 5.75 by using 4 M HCl to precipitate the proteins. The precipitated proteins were centrifuged (13 000g) at 4 °C for 10 min. The precipitate was washed with deionized water three times followed by filtration. Finally, it was freeze-dried to obtain protein powder.

4.3. Protein Recovery and Purity. The total protein content of both the raw whole spent hen and final protein isolates was determined by a LECO (Nitrogen/Carbon) analyzer (TruSpec CN, MI, CA, USA). Raw spent hen meat and protein isolates were freeze-dried in a drier (LABCONCO Inc., FreeZone12, MO, CA, USA) and ground into fine powder before analysis. The factor 6.25 was used for converting nitrogen measurement to protein. The recovery yield was measured as difference in total protein contents of isolates and raw material using method reported by Omana et al.67

4.4. Sample Preparation. Initially, plasticizers including sorbitol, ethylene glycol, poly(ethylene)glycol, glycerol, and butanediol were scanned to find out interaction of plasticizers with protein. The blends of protein with plasticizers (10, 15, 20, 25, 30, and 35%) were prepared and compression molded. Mostly nonhomogeneous and too brittle films were obtained with all other plasticizers except glycerol; therefore, glycerol was chosen and investigated further. As protein-based films have low mechanical strength, so the study was focused to increase the tensile strength of protein films and only films with better mechanical strength were further studied. Glycerol was found better plasticizer with these proteins; therefore, only films prepared with glycerol are reported here. Different blends of spent hen proteins were prepared. The blends using glycerol, chitosan, and bentonite were prepared using a laboratory blender for 30 min. Blends were sealed in a plastic bag and kept at room temperature for 72 h to give maximum time for nanoparticles to interact with the proteins.

4.5. Film Preparation. Films of plasticized materials were prepared by compression molding the resin for 10 min at 120 °C and 3500 psi pressure using a carver press.

4.6. Film Thickness. Digital caliper (Digi-Max Caliper, Sigma-Aldrich, USA) was used to measure thickness and width of the films at three different places, and the values were averaged. The average film thickness was used for the determination of mechanical properties, dynamics mechanical properties, and WVP.

4.7. Thermal Property Measurement. The thermal stabilities of the extracted spent hen protein powder and derived films were studied using TGA Q50 (TA Instrument, USA) under a continuous nitrogen flow between 25 and 600 °C at a heating rate of 10 °C/min. The denaturation behaviors and phase transitions were investigated using a calorimetric apparatus (3920 Modulated DSC, TA Instrument, USA) in a continuous flowing nitrogen atmosphere. The instrument was calibrated for heat flow and temperature using a sample of pure indium. All samples were scanned at a heating rate of 5 °C per minute in a temperature range of 25−300 °C.

4.8. Mechanical Property Measurement. Tensile properties (tensile strength and elongation to break) of the films were measured in triplicate at room temperature using a universal testing machine (Autograph AGS-X Shimadzu, Canada) following ASTM standard method D822. The averaged specimen dimensions were 50 mm × 9 mm × 0.4 mm (length × width × thickness). A 50 N load cell with a crosshead speed of 0.90 mm/s was used. Before testing, samples were equilibrated at 25 °C and 65% relative humidity (RH) for 48 h.

4.9. X-ray Diffraction. XRD analyses were performed using a Rigaku Ultima IV XRD unit with Co radiation operated at 38 kV and 38 mA. The samples were scanned from 5° to 50° (2θ) in a continuous scanning mode at rate of 2°/min with a 0.02 step size.

4.10. Dynamic Mechanical Analysis. The viscoelastic properties of films were measured by using DMA Q800 (TA Instrument). The films were conditioned for 48 hours in a desiccator at 50% RH. The analysis was carried out in a tensile mode at an oscillatory frequency of 1 Hz with an applied deformation of 0.2% during heating with a temperature scan from −90 to 200 °C with a heating rate 2 °C/min. Rectangular samples of average dimensions 16 × 7 × 0.40 (length × width × thickness) were used for obtaining tan δ and storage modulus as a function of temperature (Paschoalick, 2003). Each sample was analyzed in duplicate at least and average values reported.

4.11. Transmission Electron Microscopy. TEM images of films were taken by using a TEM/STEM Morgagni 268 (Philips, Hillsboro, USA) instrument operated at 80 kV. Small piece of each film was obtained, and their ultrastructure was preserved by using fixative solution (2% glutaraldehyde, 2% paraformaldehyde and 0.1 M phosphate buffer of pH 7.3) for a week. Films were dehydrated by rinsing with series of graded ethanol (50, 70, 90, and 100%) using 20 min of rinsing for each solution. Then, infiltration of each sample was performed with an ethanol/spurr resin mixture (1:1 ratio for 2 h). The mixture was removed followed by the addition of fresh spur resin only. After that each sample was embedded in flat molds with fresh spurr resin and cured at 70 °C in an oven overnight. Film samples of 0.4 mm thickness were sectioned and picked up on a copper grid.

4.12. ATR−FTIR Analysis. FTIR (Bruker Optics, Ettlingen, Germany) equipped with a single bounce diamond ATR crystal was used to determine functional group changes of the powdered protein and prepared films. The analysis for each sample was performed in the wavelength range of 400−4000 cm⁻¹. All sample spectra were collected using 16 scans at a resolution of 4 cm⁻¹ and averaged using OPUS software version 6.5 provided by Bruker. A background spectrum of the clean ATR crystal was taken before running the sample spectrum. Spectral examination, measurements, and processing were done using Nicolet OMNIC spectra software.

4.13. Water Vapor Permeability. WVP was carried out gravimetrically using ASTM standard method E96-95 with some modifications. The film samples were first sealed on a permeation cell (exposed film area 3.14 × 10⁻⁵) containing anhydrous calcium chloride (0% RH). The cell was then
placed in a desiccator containing saturated sodium chloride solution (75% RH). The cell weight was observed after every 24 h for 6 days.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03501.

Stress and strain values of the blended films for screening and optimization of nanoclay contents and digital photographs of the obtained films (PDF)

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Notes

The authors declare no competing financial interest.

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