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Development and Characterization of a Novel Soy Protein Isolate based Composite Film Enriched with Glycyrrhizin Nanogel Particles

Aritra Sinha

SENIOR RESEARCH ANALYST, DEPARTMENT OF FOODS,DS GROUP,NOIDA,INDIA

Abstract -*This study focuses on the development and characterization of a novel biodegradable edible film made from soy protein isolate enriched with alginate-glycyrrhizin nanogel(GL-ALG NGP). Nanoparticles of particle sizes below 100 nm were synthesized using glycyrrhizin(GL), calcium chloride and, sodium alginate(SA) through the reverse micro-emulsion/ internal gelation method. Soy protein isolate (SPI) based films were prepared by a simple casting procedure by incorporating GL-ALG NGPs in SPI solution in different ratios of (SPI: GL-ALG NGPs) 5:0, 5:1, 2:1, 1:1, and 1:1.5. Glycerol was used as a plasticizer in the film-forming solution. The effects of the proportions of GL-ALG NGPs addition on the thickness, mechanical properties, water vapor permeability, UV barrier performance, antioxidant activity, and antimicrobial property of the obtained films were studied. The GL-ALG NGPs were analyzed using Dynamic Light Scattering. Microstructural studies of obtained films were performed using Scanning Electron microscopy. Results show incorporation of GL-ALG NGPs in soy protein-alginate complex produced smoother, compact, and more continuous matrices as compared to pure SPI films. The test results indicated that blending of SPI with GL-ALG NGPs in the ratio 1:1 increased tensile strength of obtained films by 185%, reduced water solubility to 23.59%, and water vapor permeability to 0.3087 g-mm/m2-d-kPa.Obtained films exhibited good UV barrier performance, antioxidant activity and inhibited the growth of E.coli, S aureus, Enterobacter sakazakii, and A.niger. So, soy protein isolate-based films enriched with GL-ALG NGPs are active biodegradable edible films that can be used to extend the shelf life of food products.*

Keywords: **Biofilm, soy protein, biodegradable, glycyrrhizin, active packaging, nanogel, microemulsion.**

1. INTRODUCTION

A current trend in food packaging development is that, where ever possible, packaging films should not only be natural and environmentally friendly but also functional and cost-effective. Single-use plastics pose threat not only to the environment but also to terrestrial and aquatic life [1]. This has necessitated the development of active edible films made from natural biodegradable materials. Edible films are generally defined as continuous structures that can be prepared from edible materials, such as proteins, polysaccharides, and lipids. They can be used as coatings on food or between food components. They provide barrier and protection while improving the quality and safety of food products [2]. Moreover, food scientists are stressing the need to replace sugar with non-caloric natural sweeteners in edible films and coatings mainly due to the detrimental effects of sugar especially in diabetic patients [3]. This study demonstrates the development and characterization of soy protein isolate (SPI) based edible films made with sodium alginate(SA) nanogel particles loaded with glycyrrhizin(GL), a naturally occurring herbal non-calorific sweetener with numerous physiological benefits.

Protein-based edible films have become quite significant in recent years due to their texture and functional properties. Soy protein in particular has been incorporated in edible films due to its nutritional and functional benefits. They are abundant, cheap, and biodegradable which makes them ideal to be developed into films [4]. Due to the presence of both polar and non-polar side chains, soy protein molecules exhibit strong intra and intermolecular interactions which increase their yield point, tensile strength, and mechanical properties [5]. However, films made from soy protein have limited commercial applications due to their inferior mechanical strength and poor moisture barrier properties. Several methods have been studied to enhance barrier properties of soy protein-based films such as the introduction of hydrophobic compounds like lipids[6] and polysaccharides into the film-forming solution, modifying the protein network through the addition of cross-linking agents like glutaraldehyde, etc [7]. Recently, the incorporation of nanoparticles, nano-fillers. etc into protein matrix have attracted growing interest for enhancing mechanical strength and thermal stability of films [8].

This study focuses on enhancing the mechanical and barrier properties of a SPI-based edible film made from a multi-component system consisting of glycyrrhizin alginate nanogel particles (GL-ALG NGPs). Dispersion of GL-ALG NGPs into the SPI matrix formed a continuous and cohesive network stabilized by hydrogen bonds and intermolecular electrostatic interactions. Composite films made from protein and polysaccharides have been studied the least mainly because mixtures of proteins and polysaccharides are unstable due to the large molecular size of alginate molecules and weak intermolecular interactions between protein and alginate. Phase separation is the typical phenomenon observed in protein-polysaccharide mixtures because of thermodynamic incompatibility between two macromolecules [9]. Such problems can be overcome by modifying structures of film-forming materials like polysaccharides (eg. forming nano-sized particles) for better compatibility. The macromolecular interactions between proteins and anionic polysaccharides were explained by *Samant*

et al,1993 [10].In 1994, Shih reported the addition of polysaccharides to soy protein to improve the water stability of films [11]. The rheology and water adsorption properties of alginate-soy protein complexes were reported earlier [12]. However, composite film systems containing soy protein isolate and alginate nanogel particles with improved properties have not been studied yet. The purpose of the research was to evaluate the properties of edible films made from soy protein isolate fused with GL-ALG NGPs.

Glycyrrhizin is a unique product extracted from the roots of *Glycyrrhiza glabra,* commonly known as licorice. It is used as a food additive(E958) because of its very high sweetness(30-50 times as sweet as sucrose) [13]. Structurally, glycyrrhizin is a type of triterpene saponin used as an emulsifier [14] and gel-forming agent in foods, beverages, and cosmetics [15]. It is a direct inhibitor of HMGB1 with anti-inflammatory [16], anti-cancer [17], anti-diabetic activities [18]. Glycyrrhizin also inhibits the growth and cytopathology of several unrelated DNA and RNA viruses [19]. Despite its numerous benefits, its usage in food applications is limited due to its intense woody licorice aftertaste. An overdose of glycyrrhizin in humans may show side effects such as hypokalemia and salt retention [20]. Moreover, glycyrrhizin is poorly soluble in water and biological fluids, readily precipitate out at acidic conditions, and undergoes photochemical degradation [21], thereby decreasing its bioavailability and functional properties under conditions of environmental stress. Previously, biodegradable materials like chitosan and gum katira have been used as encapsulating agents to protect glycyrrhizin from degradation under environmental stress and enhance its bioavailability for targeted drug delivery [22]. However, the lack of pH responsiveness of these materials cannot protect GL from strong acidic fluids. To overcome such problems, various modifications and preparations of glycyrrhizin have been studied in the field of pharmacology and medicine in recent years, which include developing glycyrrhizic acid nano-particles [23], glycyrrhizin liposomes [24], and glycyrrhizin mediated nanogel particles [25]. This study demonstrates a simple, inexpensive procedure of the development and incorporation of glycyrrhizin enriched alginate nanogel particles into soy protein-based edible films for improved characteristics where GL acts as a bioactive compound.

Sodium alginate, a FDA-approved anionic biopolymer that is non-toxic, easily available, cheap, and biodegradable has been used to synthesize GL nano-particles. However, being highly hydrophilic, alginate-based films are readily soluble in water, a property undesirable for designing edible films [26]. To overcome this, the gelation of alginate is performed in the presence of divalent metal ions, like calcium ions [Ca^{2+}], a phenomenon which is explained by the generally known "egg-box" model to inhibit its solubility [27]. In this research, glycyrrhizin is encapsulated in sodium alginate, a pH-responsive biodegradable material using a reverse microemulsion method combined with internal gelation by Ca^{2+} ions.GL-ALG NGPs were obtained through ionic interactions among carboxyl groups of alginate, glycyrrhizin molecules, and Ca^{2+} ions. The stability of nanogel particles was enhanced and smaller-sized particles were obtained by intermolecular hydrogen bonds between glycyrrhizin and alginate. Dynamic light scattering of obtained nanogel particles showed an average particle size of 61.37 ± 0.63 nm.

A simple casting method was performed for developing the films. A control film was developed without the incorporation of GL-ALG NGPs for comparison. Although there are studies available showing the development of edible films using natural sweeteners like *stevia rebaudiana,* glycyrrhizin provides a better alternative because it not only acts as a viable sugar substitute but also modifies the physical and functional properties of edible films. Antimicrobial soy protein-based films containing licorice reside extract has been studied before [28]. However such films had poor barrier properties and texture with high swelling degrees limiting their application in packing high moisture-laden foods. Mechanical tests of obtained edible films revealed that films with more tensile strength, higher solubility, and reduced water vapor permeability were obtained with the addition of GL-ALG NGPs. Moreover, developed films exhibited radical scavenging activity and inhibited microbial growth of food-borne pathogens like *Enterobacter sakazakii*, *E.coli, S aureus*, and *A.niger*. Hence, the incorporation of GL-ALG NGPs into soy-based films served multiple purposes. It improved the mechanical and barrier properties of obtained films and could extend the shelf life of food products due to its antioxidant activity. Additionally, such natural biodegradable films can be used to pack foods where sugar from the packaging contents could be substituted by a natural sweetener from its composition, a property beneficial to diabetic patients.

2. MATERIALS AND METHODS

2.1 Materials

Glycyrrhizin-alginate nanogel was obtained from sodium alginate(>98%), pure mono ammonium glycyrrhizate ($C_{42}H_{65}NO_{16}$, 839.98 g mol^{-1}, ≥99%), calcium chloride dihydrate($CaCl_2.2H_2O$), liquid paraffin oil, Span 80, and Tween 80. Sodium alginate powder (>98%) was purchased from Bakersville India Private Ltd, calcium chloride dihydrate was purchased from Qualigens fine chemicals, Mumbai. Pure mono ammonium glycyrrhizate ($C_{42}H_{65}NO_{16}$, 839.98 g mol^{-1}, ≥99%)derivative was obtained from Sunpure Extracts Private Limited, Delhi. Edible films are developed with soy protein isolate, glycerol, HCl, and water. Glycerol was purchased from Fisher Scientific, Mumbai. Soy protein isolate powder (90%)was procured from ProFoods Pvt, Ltd, Bhiwandi. Tween 80 and Span 80 were bought from Sigma Aldrich, Darmstadt, Germany, and liquid paraffin oil was obtained from a local manufacturer.

2.2 Purification of Glycyrrhizin

Glycyrrhizin is typically available in the form of its ammoniacal salt to increase solubility. To eliminate any

effect of ammonia, we purified the substance by dissolving 25g of mono ammonium glycyrrhizate in 1500 ml de-ionized water and adjusted the pH of the resulting solution to 2.5 using acetic acid. The process was repeated thrice to ensure no ammonia was present. The solution's purity was tested spectrophotometrically for free glycyrrhizic acid at 256.5 nm. Purity was recorded to be >99.5%. The solution was freeze-dried to obtain pure glycyrrhizin powder.

2.3 Preparation of Nanogel

GL-ALG NGPs were developed using a reverse micro-emulsion /internal gelation method (Fig.1) reported earlier by *Zhu et al* [29]. Briefly, 0.5 ml of Span 80 and 0.15 ml of Tween 80 were dissolved in a 200 ml flask filled with 50 ml liquid paraffin (oil phase).To this,15 ml of 0.5% SA solution was added drop-wise to form a W/O emulsion under constant stirring at 1000 rpm in a water bath at 40^0C. After emulsifying the solution for 60 mins,5 ml of 0.1% $CaCl_2$ solution containing 1.5% GL solution in 60% ethanol solvent was added slowly into the W/O nanoemulsion. The emulsion was stirred at 1000 rpm at 40 ^{0}C for 1 hr to allow gelation. Nanogels were collected by centrifugation and stored in a freezer at -80 ^{0}C for further use.

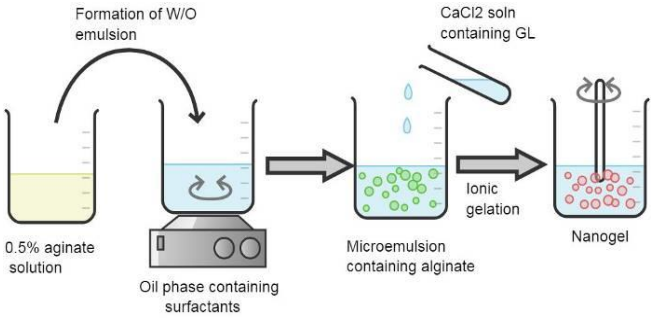


Fig- 1: Preparation of Nanogel using Micro-emulsion/internal gelation method

2.3.1. Particle size, microstructure, and zeta potential of prepared nanogel particles

Glycyrrhizin-loaded alginate nanogel particles were dispersed in water and characterized for particle size and zeta potential using dynamic light scattering with a *Zetasizer Nano Z*, Malvern Products, New Delhi.

The GL-ALG NGP structure was analyzed by scanning electron microscopy(JEOL, JSM-5911LV, Tokyo, Japan)at Nishka Research Laboratory, Andhra Pradesh.

2.3.2.Encapsulation- Efficiency

For determining encapsulation efficiency, the nanogel suspension was centrifuged in a Remi Cm 8 Plus cooling centrifuge at 15000 rpm for 30 minutes at 4^0 C. The supernatant was collected and analyzed spectrophotometrically in a UV-3600i spectrophotomer (Shimadzu Company, Japan) for free glycyrrhizic acid at 256.5 nm. Encapsulation efficiency was calculated using Equation 1:

$$\%\text{Encapsulation Efficiency, EE} = \frac{[G_t - G_f]}{G_t} * 100 \quad [1]$$

where G_t denotes total glycyrrhizic acid and G_f denotes free glycyrrhizic acid.

2.4. Film Preparation

The membranes were obtained by casting method, using five different proportions of alginate-nanogel particles and keeping the quantity of plasticizer and soy protein fixed in all formulations according to Table 1.

Film	Soy-Protein Isolate,g	GL-ALG NGPs ,g	Glycerol ,ml	Total volume of solution ,ml
SP1	5	0	1	100
SP2	5	1	1	100
SP3	5	2.5	1	100
SP4	5	5	1	100
SP5	5	7.5	1	100

Table 1: Recipes of film-forming solutions

In this method, 5 gm of soy protein isolate(SPI) powder (purity 90%) was dissolved in 50 ml de-ionized water and stirred continuously in a water bath at room temperature for 2 hours. To prepare heat-denatured films, the SPI solution was heated at 90^0 C for 30 minutes. Heated solutions were cooled to room temperature and the pH of the solution was adjusted to 2-3 by drop-wise addition of 1N HCl. The adjustment of the pH is to enhance soy protein solubility in an aqueous solution [30]. The solution was filtered through a cheesecloth to remove insoluble protein materials. The filtrate volume was adjusted to 100 ml using de-ionized water. To this solution, 1 ml of glycerol and different concentrations of GL-ALG NGPs in the ratio of (SPI: GL-ALG NGPs)5:0,5:1,2:1,1:1 and 1:1.5 were added under constant stirring on a water bath [31]. The amount of soy protein isolate and glycerol were kept constant in all formulations to directly investigate the effect of GL-ALG NGPs in edible films. The mixture was then homogenized at 3000 rpm for 5 min using a *T-25 digital Ultra Turrax homogenizer*, IKA India Pvt. Ltd., and heated at 90^0C on a hot metal plate for 20 minutes with vigorous stirring to prevent the formation of bubbles. The film solution was then poured, leveled, and left to dry on a silicone surface as shown in Fig. 2. at a temperature of 24^0 C for 48 hrs approximately. Once obtained, the films were equilibrated at 48 % relative humidity and 25^0 C for 48 hrs before characterization.

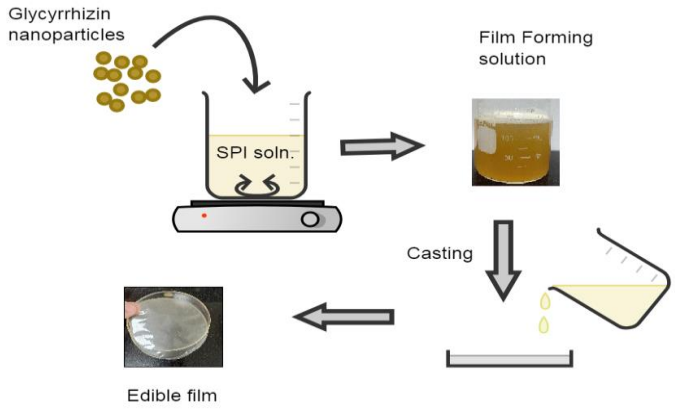


Fig- 2: Preparation of Film using the casting method

2.5. Film Thickness

The thickness of the films was measured with an *Outside Micrometer 3203-25A, Insize, India* at 5 random positions of the film with 0.01 mm accuracy. Water Vapor Permeability and mechanical properties of obtained films were calculated based on film thickness.

2.6. Optical properties

The L, a,b values of obtained films were measured using the *Hunter lab colorimeter*. The light transparency of the films was measured in a *UV-Vis Spectrophotometer* (*Shimadzu,26000, Kyoto, Japan)*.In this test, rectangular strips of film specimens (3 X 3 cm) were placed in the test cell of the spectrophotometer, and transmittance was recorded at wavelengths 200nm-800nm. All tests were conducted in triplicates.

2.7 Mechanical Properties

The tensile strength and elongation at break of the protein GL-ALG composite films were tested according to the method mentioned in STAS ASTM D882-02(Standard Test Method for Tensile Properties of Thin Plastic Sheeting) [32]. For this test, a *Tinius Olsen H1KS Benchtop Tester, ATE Corp*. was used. Tests were performed at an ambient temperature of 25 ^{0}C. In this method, three replicates of strips of dimensions 100 mm x 10 mm were cut for testing. The rate of grip separation was 10mm/minute. The parameters determined were maximum load at break (MPa) and extension of length at break (%)The tensile strength (TS) and elongation at break (E%) of each film were calculated according to equations 2 &3 :

$$TS(MPa) = \frac{\text{Maximum force at break}}{\text{Film surface area}} \quad (2)$$

$$E\% = \frac{\text{Extension of length at break}}{\text{Initial length}} * 100 \quad (3)$$

2.8. Moisture sorption Isotherm

The moisture sorption isotherm of each film was determined using the method reported by Ngo et al [33]. In this method, each film was cut into 3cm x 3 cm squares and dried in a hot oven at 105^{0}C for 3 hours. The dried films were then placed in desiccators containing dried silica gel for 48 hours. The samples were then placed in several different desiccators containing saturated salt solutions to maintain constant relative humidity at 11%,33%,53%,65%,75%, and 86%. A ball of cotton wool soaked in formic acid was used as an antifungal agent in desiccators with high relative humidity. The samples were weighed daily until the weight change did not exceed 0.1% after being weighed three consecutive times. The equilibrium moisture content, EC(g water/100g dry solid) of the obtained films at each relative humidity was calculated using equation 4,

$$EC = \frac{W_e}{W_i}(M_i+1) - 1 \quad (4)$$

where w_e is the equilibrium weight of the film sample(g),w_i is the initial weight of the film sample (g), and M_i is the initial moisture content of the film sample (g water/100g dry solid). All measurements were performed in triplicates.

2.9. Water Vapor Permeability (WVP) and Water Vapor Transmission Rate (WVTR)

The WVP of prepared films was measured according to the gravimetric method at 25^0±1^{0}C following the ASTM method E96 (2000). This method was described in detail by Rachtanapun [34]. In this method, a sample of the edible film was firmly fixed on top of a container filled with silica gel. The sealed containers were then placed in desiccators previously saturated with sodium chloride at a temperature of 25 ^{0}C. The films were weighed daily for 10 days and the WVP of films was calculated according to the formula (5).

$$WVP = \frac{W \times T}{t*a*P*(R_1 - R_2)} \quad (5)$$

where 'WVP' is Water Vapor Permeability (gH_2O mm.), 'W/t' is the constant rate of weight change, 'T' is the average thickness of the film (mm), 'a' is the permeation area(cm^2), P is the partial pressure of water vapor at 25^0 C(3.159 kPa) and(R1-R2) is the relative humidity difference between two sides of the film.

The WVTR of the obtained films was determined from the slope of the straight line (g/day) divided by permeation area(m^2). All measurements were done in triplicates.

2.10. Microstructure

The microstructure of the obtained films was studied under a Scanning electron microscope (SEM)(JEOL, JSM-5911LV, Tokyo, Japan)at Nishka Research Lab, Andhra Pradesh. The results were noted after observing at least 10 different areas.

2.11. Antioxidant property

The radical scavenging activity of obtained films was determined using DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay [35]. The stable free radical DPPH exhibits a deep violet color in solutions and shows strong absorption at 517 nm. The deep violet color disappears when an electron is paired off by an antioxidant. The decrease in absorption is a measure of the antioxidant activity. In this method, 3 ml of edible film solution was mixed with 1mL of 1 mM of DPPH dissolved in methanol. The mixture was mixed vigorously in a vortex shaker and left in the dark at ambient temperature for 30 minutes. The absorbance was then measured at 517 nm. The sample solution acts as a hydrogen atom donor and on reaction with the DPPH solution forms a stable non-radical form of DPPH with simultaneous change of the solution from violet color to pale yellow Control sampleS were prepared using the same method without glycyrrhizin. The percentage of DPPH free radical quenching activity was determined using the following equation 6:

$$\text{DPPH scavenging effect,\%} = \frac{\text{AbsDPPH} - \text{AbsExtract}}{\text{AbsDPPH}} * 100$$

where AbsDPPH is the absorbance value at 517 nm of the methanolic solution of DPPH and AbsExtract is the absorbance value at 517 nm for the sample extracts. Each sample was assayed at least five times.

2.12. Film solubility in water

Film solubility was determined using a method from Jutaporn *et al*. In this method, film portions were cut measuring 1×3 cm^2 were cut were dried at 110˚C in a vacuum oven for 24 hrs. Portions were weighed to the nearest 0.0001 g for the initial dry weight. Films were then immersed in a 100 ml round bottom flask containing 50 ml of distilled water and shaken gently in a rotary shaker at 25 ˚C for 24 h. The solution obtained was then filtered through Whatman No. 1 filter paper to recover the remaining un-dissolved film. The remaining pieces of film portions after dissolution were dried at 110˚C to obtain a constant weight (Final dry weight) [36]. Tests for each type of film were carried out in three replicates. Solubility in water (%) was calculated by using Equation 7:

$$\text{Solubility \%} = \frac{\text{Initial dry weight} - \text{Final dry weight}}{\text{Initial dry weight}} * 100 \quad (7)$$

2.13 Evaluation of Antimicrobial activity

Edible films must be microbiologically safe for human consumption. For this purpose, the antimicrobial activity of all the film-forming solutions was tested against enterobacteria, *Escherichia coli*, *Staphylococcus aureus*, yeasts, and molds using the agar well diffusion method reported by Ngo. [33]

2.15. Statistical Evaluation

All the experiments were conducted in triplicates and the results were expressed as mean value ± standard deviation (SD). Statistical analysis and visualization were performed using STHDA(Statistical tools for high-throughput data analysis). Comparisons among multiple groups were determined using a one-way analysis of variance. (ANOVA)

3. Results and Discussions

3.1. Characterization of nanogel

3.1.1 Particle size and zeta potential

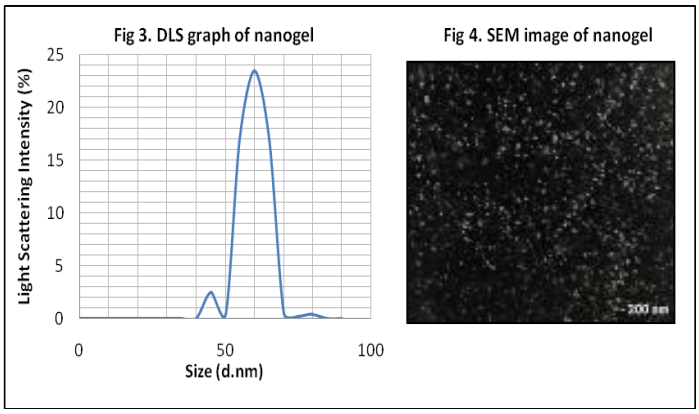
The results of the mean particle size and zeta potential are shown in Table 2. The negative surface charge is due to carboxyl groups of ALG. Overall the negative surface charge of nanoparticles can enhance electrostatic interactions with positively charged soy protein isolate molecules developing a compact structure [37]. Fig 3 shows the DLS results of obtained nanoparticles.

Table 2: Particle Size and Zeta Potential of GL-ALG NGPs

Sample	Z-average(d.nm)	Zeta Potential (mV)
GL-ALG NGPs	61.37 nm	-43.77

3.1.2 Scanning Electron Microscopy

The microstructure of the nanogel was studied by SEM at and the obtained micrograph of the GL-ALG NGPS (Figure 4)shows that the nanoparticles obtained were nearly spherical and uniformly distributed.



3.1.3.Encapsulation efficiency

The encapsulation efficiency of glycyrrhizin sodium alginate was determined using the spectrophotometric method. Calculations were done in triplicates. The mean encapsulation efficiency recorded was 87.45± 0.25%.

3.2. Thickness and Mechanical properties

The obtained films were soft, smooth, shiny, and flexible without visible pores or cracks in the structure. The films had no odor but a sweet taste. The films showed low adhesion to the silicone surface with regular edges. The thickness and mechanical properties of the obtained films are shown in Table 3.

It is observed that the thickness of the films increases slightly with the addition of GL-ALG NGPs into the film-forming solution. Films made without GL-ALG NGPs had a minimum thickness of 61.2 ± 2.17 µm. The thickness increases by increasing the content of GL-ALG NGPs from SP1 to SP5, with SP5 recording a maximum average thickness of 77.2 ± 2.46 µm. The increase in thickness may be attributed to the weak electrostatic interactions between protein and alginate molecules. Also, increasing alginate concentration may increase electronic repulsion between negatively alginate molecules resulting in a reduction in particle aggregation and increased film thickness.

Mechanical properties i.e Tensile strength (TS) and elongation at break (E%) of the obtained films are shown in Table 3. It is observed that the tensile strength of obtained films without GL-ALG NGPs was 3.67 ±0.21 MPa and increased to a maximum value of 10.46 ± 0.01 MPa in SP4 when GL-ALG NGP concentration increased by 5%.On further increasing the GL-ALG NGP concentration to 7.5% in SP5, a drop in tensile strength to 6.46 ± 0.1 MPa was noted. The increased tensile strength(TS) of obtained films may be due to cross-linking and ionic interactions between amino groups of soy protein and carboxyl groups of GL-ALG NGPs as reported by Shih et al(1994) [11]. The iso-electric point of soy protein is between 4 to 5. At pH values lower than 4.0 the positively charged protein molecules bind with the negatively charged GL-ALG NGPs.Additionally, the formation of hydrogen bonds between active groups of protein and the phenolic hydroxyl group of GL resulted in a stable compact structure. The drop in tensile strength in SP5 may be due to intermolecular electronic repulsions among protein chains and alginate molecules or due to steric hindrances between hydroxyl groups of alginate and amino groups in protein chains.

Percent elongation at break(E%) of the obtained films is shown in Table 3 . It is observed that the elasticity of films decreased slightly with an increase in the concentration of GL-ALG NGPs. The decrease in elasticity may be attributed to the uneven interactions between the protein layer and GL-ALG nanoparticles.

Film	Thickness, µm	Tensile strength, MPa	Elongation,%
SP1	61.2 ± 2.17	3.67 ±0.21	24.57 ± 0.89
SP2	68.5 ± 1.89	5.50 ±0.31	17.1 ±1.19
SP3	68.7 ± 2.1	8.22 ± 0.19	13.59 ± 1.42
SP4	75.9 ± 1.49	10.46 ± 0.01	11.36 ± 2.29
SP5	77.2 ± 2.46	6.46 ± 0.1	11.17 ±2.21

Table 3: Mechanical Properties of films

The change in mechanical properties of the obtained with change in concentration of glycyrrhizin nanoparticles are shown in Figure 5

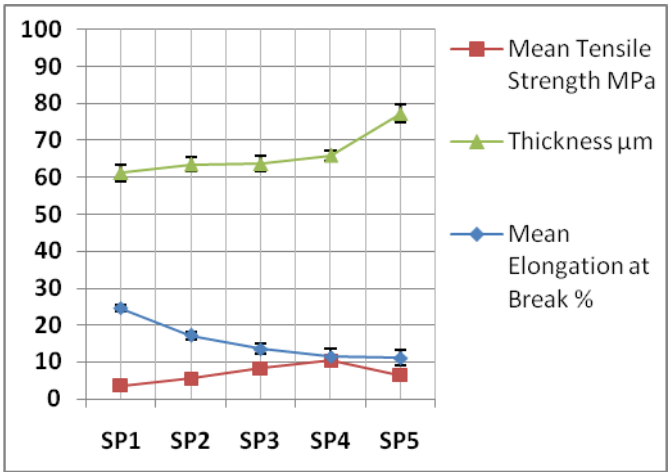


Fig- 5: Mechanical Properties of Edible films

3.3 Hydration properties

Table 4 shows the Solubility %, Water vapor permeability (WVP), and Water vapor Transmission Rate (WVTR) of the obtained edible films. It is observed that the solubility of films decreases by increasing the concentration of GL-ALG NGPs into the film-forming solution. The minimum solubility was observed in film SP5. The obtained films prepared with GL-ALG NGPs retained their integrity even after 75 minutes of being immersed in water at ambient temperature(25 ± 2^0C). The reduction of solubility is the combined result of electrostatic interactions and cross-linking between protein and alginate molecules. Such films can be used for extending the shelf life of a wide range of food products and ingredients.

It is observed that the WVP and WVTR values of the edible films initially increases on increasing the GL-ALG NGPs concentration from 0% to 5%(w/w) and fall on further increasing the concentration to 7.5% of the total film-forming solution.SP4 film has the lowest WVP and WVTR making it an ideal film to be used for packing foods with high water activity. The recorded values are lesser than the WVP, WVTR values of soy protein-PGA composite films studied by Rhim et al [38]. This shows the addition of GL-ALG nano-particles enhances intermolecular interactions among protein chains, alginate, and glycyrrhizin molecules producing more compact structures. The cross-linking reduces hydrophilic sites along protein chains and inhibits water diffusion across films. The increase in WVP in SP5 may be due to partial rupture of protein structure by hydrophilic alginate molecules increasing interstitial spaces in a protein matrix, allowing increased diffusion of water molecules through the films.

Film	Solubility,%	WVP,g-mm/m2-d-kPa	WVTR,g/m2-d
SP1	33.95 ±1.18	5.15 ± 0.0365	41.65 ±0.63
SP2	31.50± 1.98	0.4339 ± 0.0089	11.33± 0.39
SP3	27.40 ±1.66	0.4281 ± 0.0023	9.84±0.29
SP4	23.59 ± 2.19	0.3087 ± 0.0017	7.73±0.83
SP5	21.21 ± 2.19	0.3713 ± 0.0061	10.77±0.45

Table 4: Hydration properties of films

3.4. Moisture Sorption Isotherm of films

The moisture sorption isotherms of the obtained films with different concentrations of GL-ALG NGPs are shown in Figure 6. Obtained films prepared with GL-ALG NGPs (SP2 to SP5) were observed to have less water intake capacity than SP1 at each relative humidity value at 25^{0}C. At each relative humidity, water absorption intake decreases slightly with an increase in GL-ALG NGP content. Thus the hydrophilicity of obtained films decreases on the incorporation of GL-ALG NGPs which is in good agreement with the solubility and WVP values.

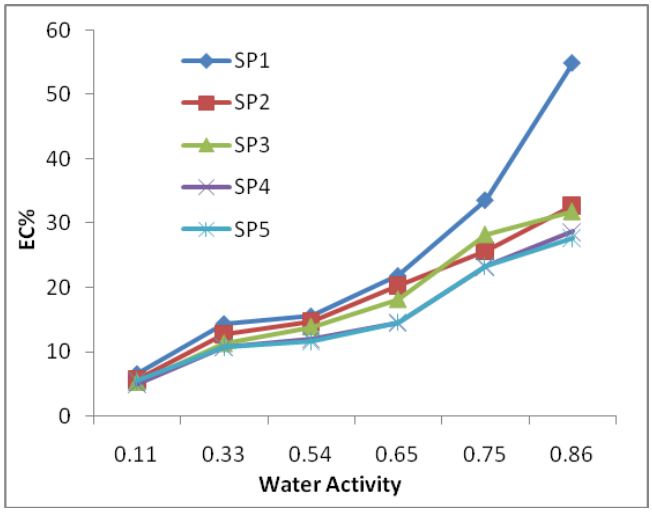


Fig- 6: Moisture sorption isotherms of films

3.5 Color and Optical characteristics of films

3.5.1.*Color*

The color and transparency of films are important factors for consumers' acceptance. The results of the color evaluation of the obtained films are shown in Table 5. The a* value shows the color of the films changes from red to green as the a* value decreased from SP1 to SP5. An increase in b* indicated films becoming more yellow probably due to flavonoids present in glycyrrhizin. The L* parameter was used to describe the brightness of the films ranging from 0 to 100. The L* values show that the concentration of GL-ALG NGPs had a negligible effect on the brightness of films as values were not significantly different.

3.5.2.*Visual characteristics*

Films were transparent, shiny in appearance, with no visible pores or crevices. While film SP1 was less smooth and had a wrinkled surface, film SP5 was slightly rough and thick. All obtained films had regular edges with no particle deposition observed on surfaces.

3.5.3. *UV-Visible light barrier performance*

Photochemical degradation of food products can lead to oxidation, nutrient losses, and the development of off-flavors [39]. UV-visible light barrier performance is thus an important parameter of edible films to pack food products that are sensitive to light. Fig.7 shows that the addition of GL-ALG NGPs improved the UV-visible light-blocking performance of edible films. The UV transmission in the range 200-400 nm was close to zero in the GL-ALG NGPs incorporated films and decreased with an increase in the concentration of nanoparticles. The same trend was observed in the visible range of 400-800 nm. GL-ALG NGPs enriched films had lower transmission than pure SPI film. This can be attributed to the UV-visible light absorption by the phenolic compounds present in glycyrrhizin.

Film	L*	a*	b*
SP1	76.24	10.14	13.08
SP2	75.96	6.64	14.22
SP3	78.51	6.59	17.59
SP4	76.12	5.57	21.12
SP5	75.27	5.08	33.34

Table 5 :L,a,b values of films

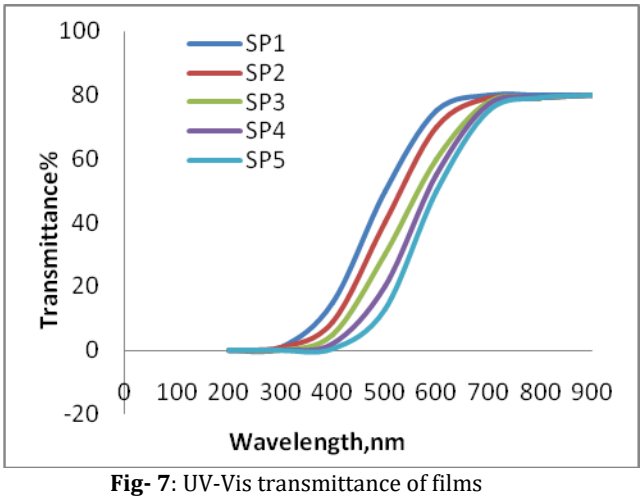


Fig- 7: UV-Vis transmittance of films

3.6 Microstructure of films

SEM images of the obtained films (Fig. 8) show that films developed with glycyrrhizin alginate nanogel particles are smooth, elastic, compact, and without pores. Films SP1 and SP5 had irregular surfaces with very small pores. Film SP5 was slightly rough with some particles deposited on its surface.SP1 had high porosity area (pore diameter 4.56-12.2 nm and depth 136-297 nm).

SAMPLE	APPEARANCE	MICROSTRUCTURE
SP1 Porous and wrinkled surface with heterogeneous and irregular structure		
SP2 Transparent homogeneous structure without pores and cracks, smooth and without insoluble ingredients		
SP3 Smooth homogenous structure, no pores or cracks, transparent with no particles on surface		
SP4 Smooth,regular surface with no pores or cracks.		
SP5 Slightly porous and thick membrane with particles deposited on surface		

Fig- 8 : Appearance and Microstructure of films

3.7. Applications

Edible films enriched with glycyrrhizin-alginate nanoparticles (Fig.9)can be used to pack a range of food products and ingredients like biscuits,tea leaves,ready to eat cereals,medicines.etc (Fig 10).

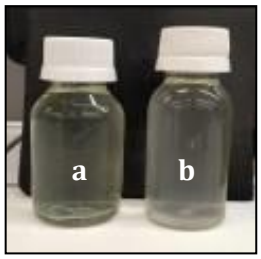


Fig- 9: a)Glycyrrhizic acid b)Glycyrrhizin nanogel

Fig- 10:Applications of films

Table 6 represents the Pearson correlation matrix between the physical and mechanical characteristics of the obtained films. It shows that some parameters like thickness is positively influenced by parameters like Tensile strength and antioxidant activity etc.($p<0.05$).It is important to note that tensile strength and solubility of films are negatively influenced by WVP($p<0.05$).Furthermore, we can observe a positive correlation between solubility and elongation at break.

	T	TS	E	L	a	b	W	S	A
T	1								
TS	0.69*	1							
E	-0.94	-0.8	1						
L	-0.33	0.27	0	1					
a	-0.95	-0.7	0.97*	0.14	1				
b	0.83	0.29	-0.72	-0.42	-0.71	1			
W	-0.8	-0.7	0.91	-0.07	0.96	-0.47	1		
S	-0.95	-0.68	0.92	0.2	0.87	-0.9	0.69	1	
A	0.88	0.73	-0.98	0.06	-0.98	0.65	-0.97	-0.83	1

Table 6:Pearson correlation matrix between physical,mechanical and functional properties

T:thickness; TS:tensile strength; E:elongation; L:brightness; a:red-green axis; b: yellow-blue axis; W: water vapor permeability; S: solubility; A:antioxidant activity
*Correlation significant at the 0.05 level

3.8 Antioxidant property

The antioxidant activity is an important parameter of edible films to prevent oxidative degradation, such as lipid peroxidation in foods [40]. An antioxidant can reduce the stable radical DPPH to yellow colored diphenyl-picrylhydrazine(DPPH). Mainly flavonoids present in licorice roots is responsible for the radical scavenging activity in glycyrrhizin. Table 7 reveals the radical scavenging percentage of each sample . Tocopherol and trolox were

used as standards. Sample SP5 reported the maximum scavenging ability at 48.5 ± 0.9 %,while sample SP3 and SP4 had comparable radical scavenging ability of 43.8 ± 1.7% and 44.2 ± 2.1 % respectively. No color change was reported in Control film SP1,while sample SP2 reported scavenging capacity of 36 ±3.2 %(Fig 12) .This shows obtained films can prevent oxidation of its contents making them an ideal packaging material for extending shelf life of foods and beverages.

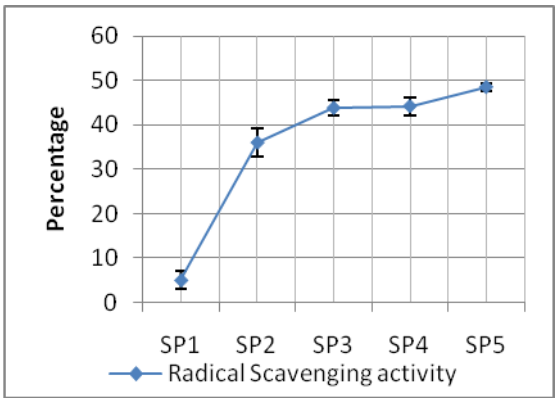


Fig-11 : Radical scavenging activity %

Material	DPPH Scavenging Activity
SP1	5± 2
SP2	36± 3.2
SP3	43.8 ± 1.7
SP4	44.2 ± 2.1
SP5	48.5 ± 0.9
Trolox	55.9 ± 2.4
α-Tocopherol	61.3 ± 3.3

Table 7: Radical scavenging % values

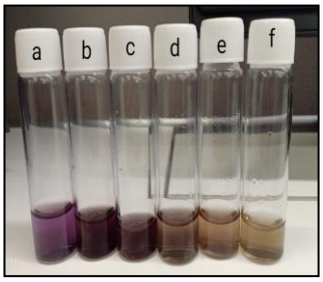


Fig -12: Color of DPPH solutions on addition of film-forming solutions a) Control b) SP1 c) SP2 d) SP3e) SP4 f) SP5

3.9.Antimicrobial activity of film forming solution

The antimicrobial action of the soy protein-GL-ALG composite film forming solution were tested against selected pathogens *like Enterobacter sakazakii*, *E.coli, S aureus* and *A.niger*. The zone of inhibition of the film-forming solutions against the growth of selected micro-organisms are shown in Figure 13. .The diameter of zone of inhibition indicates the antimicrobial activity of film-forming solutions.

It was observed that film SP1 did not show any inhibitory effect against the four tested micro-organisms, hence it has not been showed in the graph. The observed values show that the inhibitory effect increased on increasing the concentration of GL-ALG NGPs. This shows that glycyrrhizin nanoparticles exhibited antimicrobial activity due to its bioactive components, high surface charge and small size. Also the inhibitory effect of film forming solutions were better on E.coli and A.niger than enterobacter and S.aureus

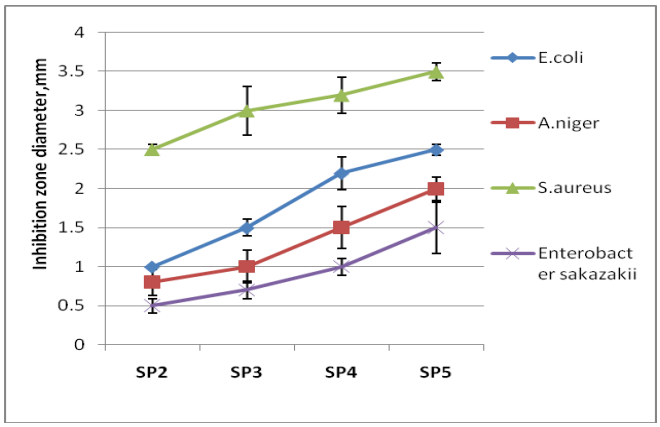


Fig- 13: Zone of inhibition of film forming solutions

4. CONCLUSION

Edible films and coatings made from nature hydrocolloids and proteins sources have replaced conventional plastic based packaging. In this work, films composed of soy protein isolate enriched with glycyrrhizin nanogel particles were obtained. The incorporation of nanoparticles in film forming solution led to an improvement of moisture barrier properties, hydration properties and mechanical properties. The edible material acts as a substitute to films made from sugar which can be beneficial to patients suffering from diabetes. Additionally, the films exhibited radical scavenging activity which can be used to prevent lipid oxidation of its contents thereby extending shelf life of foods. Besides, its physiological benefits, the obtained films have been observed to modify flavor and texture of its contents which is an important parameter for designing functional foods. The material obtained from mixing soy protein isolate and glycyrrhizin nanogel in the ratio 1:1 had optimal characteristics for use as a packaging material : low solubility, homogeneity, regular cuts and margins, low roughness ,good tensile strength, good barrier properties and elasticity. The material thus obtained can easily be reproduced on an industrial scale as the process involves use of cheap, natural, biodegradable materials and without use of rigorous automation.

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BIOGRAPHIES

Author
Aritra Sinha, Senior Research Analyst, Department of Foods, DS GROUP. Pursued BE in Food Technology from Jadavpur University, Kolkata.