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Fabrication, nanomechanical characterization, and cytocompatibility of gold-reinforced chitosan bio-nanocomposites

Nimitt G. Patel^{a,b}, Ajeet Kumar^c, Veroni N. Jayawardana^d, Craig D. Woodworth^e, and Philip A. Yuya^{a,*}

^aDepartment of Mechanical and Aeronautical Engineering, Clarkson University, Potsdam, NY, 13699, USA

^bMaterials Science and Engineering PhD Program, Clarkson University, Potsdam, NY, 13699, USA

^cCenter for Advanced Materials Processing, Clarkson University, Potsdam, NY, 13699, USA

^dDepartment of Mathematics, Clarkson University, Potsdam, NY, 13699, USA

^eDepartment of Biology, Clarkson University, Potsdam, NY, 13699, USA

Abstract

Chitosan, a naturally derived polymer represents one of the most technologically important classes of active materials with applications in a variety of industrial and biomedical fields. Gold nanoparticles (~32 nm) were synthesized *via* a citrate reduction method from chloroauric acid and incorporated in Chitosan matrix. Bio-nanocomposite films with varying concentrations of gold nanoparticles were prepared through solution casting process. Uniform distribution of gold nanoparticles was achieved throughout the chitosan matrix and was confirmed with SEM. Synthesis outcomes and prepared nanocomposites were characterized using SEM, TEM, EDX, SAED, UV-vis, XRD, DLS, and Zeta potential for their physical, morphological and structural properties. Nanoscale properties of materials under the influence of temperature were characterized through nanoindentation techniques. From quasi-static nanoindentation, it was observed that hardness and reduced modulus of the nanocomposites were increased significantly in direct proportion to the gold nanoparticle concentration. Gold nanoparticle concentration also showed positive impact on storagemodulus and thermal stability of the material. The obtained films were confirmed to be biocompatible by their ability to support growth of human cells *in vitro*. In summary, the results show enhanced mechanical properties with increasing gold nanoparticle concentration, and provide better understanding of the structure–property relationships of such biocompatiblematerials for potential biomedical applications.

Keywords

Gold nanoparticles; Chitosan; Nanocomposites; Nanoindentation; Mechanical properties

*Corresponding author. Tel.: +1 315 268 2205; fax: +1 315 268 6695. pyuya@clarkson.edu (P.A. Yuya).

1. Introduction

Chitosan, a naturally derived polymer represents one of the most technologically important classes of bioactive materials with applications in a variety of industrial [1] and biomedical [2,3] fields. Fundamental properties such as excellent biocompatibility and biodegradability make chitosan a very smart material for biomedical applications [4,5]. Chitosan is commercially obtained by deacetylation of chitin; which is a structural component of shell-fish crust [6]. It has unique structural and physicochemical characteristics that differ considerably from typical synthetic polymers. Being biocompatible, chitosan has many applications such as implants [7], biomedicines [8], dental materials [9], bone substitute materials [10], wound healing [11], tissue engineering [10], absorbable sutures [12], drug delivery [6,13,14], food packaging [15], water treatment [16], reverse osmosis [17], cosmetics [18], and many more. Chitin and chitosan derivatives demonstrate excellent performance while being used in artificial organs in the areas of skin [2,19], bones [20], nerves, cartilage and blood vessels [19].

For certain critical applications, the need for modification of physical and mechanical properties of chitosan for strength and to make it selectively responsive to certain stimuli or to immobilize biomolecules is essential. Incorporation of nanostructured materials in chitosan improves its mechanical and physical properties [14,15,21–28]. Chitosan films have been modified with various nanostructures such as carbon nanotubes [29], metallic nanoparticles, vesicles [30], and even viruses [31]. Many biomedical applications require nanostructures with low-toxicity, biocompatibility, and stability with high preferential accumulation in the target tissue or organ. Gold nanoparticles (AuNPs) have attracted enormous attention due to their nanoscale size, oxide-free components, bioconjugation, biocompatibility, and unique optical properties in applications for immunoassay [32,33], drug delivery [34], contrast enhancement [35], and thermal therapy for tumor treatment [36–38].

Limited emphasis has been given to characterization of nanomechanical properties of chitosan based nanocomposites compared to their reliability and biocompatibility characterization [28]. Chitosan based nanocomposites having heterogeneous micrometer and nanometer-sized domains [39] are time dependent materials. As a consequence changes in their viscoelastic properties can be used to explore structural variations in the film as well as fluctuations in environmental conditions. To evaluate mechanical properties which are sensitive to the state of aggregation of the filler nanostructures and the nature of the interface between the filler and the chitosan in thin films requires novel characterization tools to resolve mechanical properties on commensurate length scales. In the present study, gold reinforced chitosan bio-nanocomposite films are fabricated. The effects of nanostructure enhancement on composite morphological properties, viscoelastic properties and biocompatibility are quantified.

Gold/chitosan (Au–chitosan) nanocomposite was chosen because both gold and chitosan have good biocompatibility and have been used widely in fabrication of many devices and sensors in biomedical fields [40]. We are interested in understanding the effect of interfacial interactions between chitosan chains and the crystalline nanostructures on the mechanical

properties of the films. It is hypothesized that the thin-film mechanical properties depend on interfacial adhesion between chitosan and the filler material. Nanoscale characterization technique based on nanoindentation (NI) is used to determine the mechanical properties of the films. Quasi-static NI technique is used to measure the reduced modulus (E_r) and hardness (H) of the nanocomposites as a function of temperature and gold concentration. Dynamic nanoindentation test is used to determine the storage modulus (E'), loss modulus (E''), and loss tangent ($\tan(\delta)$) as a function of excitation frequency and also AuNP concentration. Finally, cells are cultured on both pure chitosan and chitosan bionanocomposites to access the ability of these materials to support growth of human cells *in vitro*. Samples were characterized in cell viability assays to understand if the nanostructure enhancement affects the biocompatibility of chitosan. Understanding the mechanical properties of Au–chitosan nanocomposites and their dependence on environmental conditions will aid in the fabrication of stronger and more reliable films for various applications.

2. Experimental section

2.1. Materials

Low molecular weight chitosan ($M_w = 50\text{--}190$ KDa; ~85% of deacetylation), acetic acid (99.0%), chloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) and sodium citrate tribasic dehydrate (99.0%) were purchased from Sigma Aldrich (St. Louis, MO). Ultra high purity water (resistivity $18.3\text{--}18.5$ M Ω cm at 25°C) was obtained using a Milli-Q Element® water purification system (EMD Millipore, Billerica, MA). All experiments were performed using Milli-Q water. All glassware and stir bars were first cleaned with a soap solution and rinsed with deionized water followed by bath washing in a freshly prepared aquaregia ($\text{HCl}:\text{HNO}_3$ 3:1). After which, they were thoroughly rinsed with deionized water, drained and dried in an oven at 50°C .

2.2. Preparation of Au–chitosan bio-nanocomposite films

Gold nanoparticles were obtained via the classical Terkovich et al. [41] method with slight modifications. 100 ml of HAuCl_4 (2.54mM) solution was vigorously stirred at 50°C on a hot plate stirrer for 10 min. 2 ml of freshly prepared sodium citrate (10% w/w) was quickly added to the aqueous solution of HAuCl_4 . The solution was brought to room temperature after 3 h and continually stirred for the next 12 h. Obtained gold nanoparticles were washed with DI water and subsequently centrifuged at 7000 rpm (~3500 $\times g$) for 10 min to remove the unreacted salts and finally washed Au nano-dispersion in DI water was kept in sealed glass vials for making thin films. Synthesized gold nanoparticles were characterized using SEM, TEM, UV–vis, and Zeta potential to evaluate their stability and size distributions before being dispersed in chitosan/acetic acid solution for the preparation of bio-nanocomposite films.

Low molecular weight chitosan (2% w/w) was mixed with 150ml solution of diluted acetic acid (1% w/w). The mixture was stirred at room temperature for 24 h until it turned transparent showing that chitosan had been dissolved completely. After that, the solution was filtered with a small pore sized (5 μm) filter paper and stored at 4°C in amber vials for

the later procedure. Gold nanoparticle reinforced chitosan nanocomposites were prepared by slowly adding gold suspensions of different concentrations into the chitosan solution while vigorously stirring. The mixtures were stirred for 12 h to ensure uniform dispersion of gold nanoparticles in the chitosan matrix.

The composite solutions were again characterized using SEM, UV–vis and Zeta potential for the gold particle dispersion, entrapment efficiency and to study the aggregation of gold-nanoparticles in the chitosan matrix. Solution evaporation technique was used to fabricate the nanocomposite films [26]. Gold–chitosan nanocomposite solutions were kept in petri dishes and degassed in vacuum to remove entrapped air. The degassed composite solutions were kept at room temperature for drying. After which, the dried films were carefully removed from the petri dishes and put in a vacuum desiccator to remove moisture. Finally, the as obtained films were carefully stored in a desiccator for subsequent studies. Au–chitosan nanocomposite films with the gold nanoparticles concentration (w/v) of 0 mg/ml (pure chitosan), 0.10 mg/ml, 0.15 mg/ml, 0.20 mg/ml and 0.25 mg/ml in chitosan solution were prepared for the study.

2.3. Morphological characterization of Au–chitosan bio-nanocomposite films

The film thickness and morphology of Au–chitosan nanocomposites were assessed by transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM). The size distribution of the particles was determined from the electron micrographs using Image J® software. The degree of dispersion was assessed by comparing the particle size from SEM image and the average particle size determined by dynamic light scattering (DLS) analysis. The optical properties of nanocomposites were monitored as a function of time with a Perkin–Elmer Lambda 650 UV–Vis Spectrophotometer. Small dispersion aliquots (1.0 cm^3) removed from the reaction mixture were first diluted in a 50 cm^3 volumetric flask and then transferred in the 10-mm optical-path-length quartz cuvette. The scanning range used was from 400 nm to 800 nm while the scanning speed was 400 nm/min. The surface charge density of composite particles was measured using ZetaPals® instrument (Brookhaven Instruments). The structure of composites was investigated by X-ray diffraction (XRD) using a Bruker D8® instrument. For the diffraction pattern acquisition, the step width and period were $0.02^\circ/\text{s}$ and 1.5 s respectively, while the source, sample, and detector slits were 2 mm, 0.6 mm, and 1 mm, respectively.

2.4. Mechanical property characterization

Mechanical properties of the nanocomposite films were characterized using a nanomechanical test instrument (TI-950 Hysitron Inc.). A diamond tip with a cono-spherical geometry (tip radius = $99.56\text{ }\mu\text{m}$) was used to perform indents on chitosan–Au nanocomposites. Information from quasi-static and dynamic indentation was captured to characterize elastic and viscoelastic properties of the composite films. Considering the composite applications at temperatures higher than the room temperature (25°C) and the human body temperature (37°C), nanomechanical properties were characterized at 25°C , 37°C , 60°C and 80°C . For temperature measurements, on a specially designed heating stage, the samples were secured with compression clips in order to avoid slippage and to obtain efficient thermal conductivity. Between each temperature increase, at least 30 min

was allowed to elapse for the sample to thermally equilibrate. Before initiating the indentations, the tip was brought in contact with the sample for 5 min in order to equilibrate the temperature at the specimen-tip contact. After confirming a firm initial tip contact with the film, indentation tests were performed by raising the load to 12 mN in 1 s. Upon reaching the resulting depth, the tip was dwelled at the resulting depth for 12 s followed by dynamic loading. After the quasi-static dwell period, the cyclic force of 120 μ N was applied which resulted in the dynamic oscillation amplitude between 1 nm and 2 nm (center to peak). The frequency of oscillation was scanned from 10 Hz to 200 Hz for each indent. After completing the frequency scan, the sample was unloaded from 12 mN to 0 mN in 1 s. From the frequency scan, storage modulus, loss modulus and tan delta were determined.

There was no significant difference observed in reduced modulus values characterized from the unloading curve of quasi-static indent and dynamic indent; as a result, the reduced modulus values were determined from the unloading segment of the dynamic tests. Same protocol was followed for the high temperature nanoindentation except in high temperature tests; the tip was heated to equilibrium temperature with the sample temperature before initiating the indentation load function.

2.5. Cytocompatibility analysis of Au–chitosan bio-nanocomposite films

Primary cultures of human epithelial cells derived from cervix tissue [42] were used. The cultures were maintained in keratinocyte-serum-free medium. Epithelial cells were seeded at low density (5×10^5 cells/dish) onto sterile glass coverslips (control) or coverslips covered with specific amounts of Au reinforced chitosan bio-nanocomposite films. Coverslips were sterilized by autoclaving prior to cell culture experiments. Cell number was determined at daily intervals by photographing three microscopic regions viewed by phase contrast microscopy, and counting the total number of cells.

2.6. Statistical methods

As there is a close correlation between hardness and modulus, the latter was chosen to be analyzed statistically. Reduced modulus and storage modulus over the frequency span are characterized from a single test and it was observed that they are in proportion for each film at different temperatures. For the analysis of the mechanical property results, the reduced modulus was chosen to be analyzed using SPSS v.22 software. We used level of significance, alpha, of 0.01 for all statistical tests. A two-way (4 temperature levels: 25 °C, 37 °C, 60 °C and 80 °C with 5 concentration levels: 0 mg/ml, 0.10 mg/ml, 0.15 mg/ml, 0.20 mg/ml and 0.25 mg/ml) ANOVA with 12 replications for each treatment combination was conducted on the reduced modulus. This revealed the temperature $\times$ concentration interaction was significant, $F_{(12,220)} = 46.72 (p < 0.0000)$, indicating that temperature and concentration interact in their effects on modulus. The residuals were examined to support the assumptions of normality, constancy of error variances, and independence of error terms. The normal probability of the residuals was approximately linear supporting the condition that the error terms were normally distributed ($p = 0.119$). Through Levene's test for equality of variances, it was determined that the assumption of homoscedasticity is voided. The alpha was set to be more stringent (0.01 instead of 0.05) to avoid type 1 error as a result of non-homogeneity of variances [43]. Pairwise multiple comparisons using Tukey's test for

all five films at different temperatures were performed followed by ANOVA. The difference was significant for each pairwise case in both the treatments, which shows the mean values are distinct for each comparison for Au concentration and test-temperatures. The effect of both treatments, temperature and concentration, was significant with corresponding F-values $F_{(3,220)} = 778.63$ ($P < 0.0000$) and $F_{(4,220)} = 507.47$ ($P < 0.0000$), which indicates that the reduced modulus is significantly higher in lower temperatures and higher Au-concentration levels.

For the cytocompatibility studies, similar approach for performing two-way (3 time levels with 5 Au concentration levels) ANOVA was conducted. Optical micrographs of five different regions, on each film at each interval of time were captured using phase contrast optical microscopy. No outliers were detected in the data. The results lead us to conclude that there was a significant interaction effect between time and Au concentration $F_{(8,60)} = 30.54$ ($P < 0.000$). The main effects of time and Au concentration in the composites were significant with corresponding $F_{(2,60)} = 179.34$ ($P < 0.000$) and $F_{(4,60)} = 63.9$ ($P < 0.000$). The assumption of normality was confirmed through a normal probability plot, and the equality of variances was confirmed using Levene's homogeneity test.

3. Results and discussion

3.1. Morphological characterization

3.1.1. UV-vis analysis—The Au-chitosan bio-nanocomposites were prepared by adding different concentrations of Au-nanoparticles. UV-vis spectrophotometer was used to measure the light absorbance of the samples in the UV and visible ranges. The UV-vis overlay spectra of gold nanoparticles and chitosan-Au nanocomposites in DI water shown in Fig. 1a revealed the presence of a single broad plasmon band for pure aqueous gold nanoparticles, which shifted from 528 nm to 532 nm due to interaction with different functional groups present in the chitosan. Fig. 1b shows UV spectra of Au-chitosan composite at different concentrations of gold nanoparticles varied from 0.10 mg/ml to 0.25 mg/ml. All the spectra exhibit absorption maxima at 532 nm which is a typical plasmon band, suggesting the presence of gold nanoparticles. As the concentration of AuNP added is increased, the intensity of the absorption band increases which clearly indicates that the gold nanoparticles have been successfully incorporated in the polymeric mesh of chitosan.

3.1.2. Transmission electron microscopy—In order to evaluate the interaction and dispersion of gold nanoparticles (Au-NPs) in the chitosan-gold bio-nanocomposites, the suspension was deposited onto a copper grid and analyzed by transmission electron microscope (TEM). As observed in Fig. 2a, the Au-NPs dispersed in the chitosan matrix exhibits spherical shape. The size distribution histogram for Au-NPs revealed a mean diameter of 32.4 ± 2 nm (Fig. 2b). The distance of the lattice fringes was obtained to be 0.239 nm using HRTEM images and the selected area electron diffraction (SAED) pattern (Fig. 2c) showed single crystalline particles having face centered cubic structure of Au. The narrow size distribution and the absence of agglomerates suggest that the chitosan matrix prevents the agglomeration of Au-NPs. The stability of gold nanoparticles in the chitosan matrix can be explained as the formation of a tridimensional net involving chitosan and

AuNPs since the CH₂ group of chitosan also participates in the stabilization of nanoparticles (Fig. 3).

3.1.3. Zeta potential studies—Zeta potential measurements allow predictions about the stability of colloidal dispersions of aqueous solutions. Usually, particle aggregation is less likely to occur for charged particles with optimum Zeta potential (> 30 mV) due to electrostatic repulsions [44]. The Zeta potential values of composite particles synthesized using varying concentrations of gold nanoparticles with chitosan are shown in Table 1. The Zeta potential measurements revealed that increasing the concentration of gold nanoparticles in the composite does not contribute much effect. The sufficient positive charge ($> +60$ mV) due to coating/wrapping of the gold nanoparticles with the cationic chitosan is rendered making them electrostatically stable.

3.1.4. X-ray diffraction—X-ray diffraction was used to determine information regarding the crystallinity of the bio-nanocomposite films. The Au–chitosan bio-nanocomposite obtained with various Au-concentrations had noticeably different XRD patterns Fig. 4. The ratio between the intensities of the {111} and {200} reflections increased from 0 mg/ml to 0.25 mg/ml Au concentration in the composites (Table 2). An increasing intensity ratio ($\{1, 1, 1\}/\{2, 0, 0\}$) indicates a more pronounced long range lattice order along preferential crystal orientations due to increasing Au-concentration.

3.1.5. FE-SEM—Fig. 5 shows the FE-SEM images of pure chitosan and chitosan–Au nanocomposites with different Au-NP concentration. The images clearly indicate the increasing concentration of Au-NPs within the chitosan–Au composites. EDX analysis (Fig. 6) data shows that Au in the chitosan–Au nanocomposites is in pure elemental form. No other metal impurities are present in the composites.

3.2. Mechanical characterization

The mechanical properties of the bio-nanocomposite films were probed by nanoindentation. The results were derived by making use of Hertzian contact mechanics [45]. Fig. 7 shows representative load–displacement plots of pure chitosan and Au–chitosan nanocomposites from dynamic NI experiments at room temperature. The results reveal that gold reinforced chitosan nanocomposite films have a higher stiffness responses compared with pure chitosan films.

The influence of gold nanoparticles on the elastic modulus of chitosan film was investigated; the results are shown in Fig. 8a. The results are presented as mean $\pm$ SD (standard deviation). The reduced modulus value for the pure chitosan film (0.00 mg/ml Au concentration) was determined to be $3.04 \text{ GPa} \pm 0.12 \text{ GPa}$ at room temperature (25°C). For the film with the highest gold concentration (0.25 mg/ml), the reduced modulus is $5.22 \text{ GPa} \pm 0.13 \text{ GPa}$. The reduced modulus value for the reinforced chitosan in this case is increased by about 72%. The overall trend shows a significant decrease in reduced modulus with increasing temperature ($P < 0.01$). At a temperature of 25°C , the reduced modulus is $4.66 \text{ GPa} \pm 0.14 \text{ GPa}$, while at a temperature of 80°C , the modulus reduced to $2.92 \text{ GPa} \pm 0.22 \text{ GPa}$. These results show a more viscous response as the temperature is increased ($P < 0.01$).

The effect of gold nanoparticle reinforcement and test temperature on the hardness of chitosan nanocomposites is similar to that on modulus as shown in Fig. 8b. Similar to reduced modulus trend, sudden drop in hardness values was observed when the film with 0.25 mg/ml concentration of gold was heated to 37 °C from 25 °C. By increasing the gold nanoparticle concentration, a harder and more brittle film is formed, while an increase in test temperature makes the film more compliant.

To further elucidate the effect of gold nanoparticle addition on the mechanical properties of pure chitosan, we performed dynamic nanoindentation test to determine the viscoelastic properties of the films. Fig. 9 shows plots of (a) storage modulus, and (b) loss tangent versus frequency. The storage moduli of the Au reinforced chitosan nanocomposite films were higher than that of pure chitosan. The trend in the storage modulus is similar to that seen in the reduced modulus. For the entire frequency range considered, the storage modulus was consistently much larger than the loss modulus suggesting that both pure chitosan and Au–chitosan nanocomposite films have a more elastic response.

Fig. 10 shows plots of (a) storage modulus, and (b) loss tangent versus frequency on the film with gold concentration of 0.20 mg/ml. With increasing temperature, the storage modulus decreased, indicating a decrease in the elasticity of the nanocomposite film. The loss tangent values also showed temperature dependence (Fig. 10b).

The loss tangent values at higher temperatures were lower than those at lower temperatures, indicating that the glass transition temperature (T_g) region was not covered in the test temperature range. At the glass transition temperature, the loss tangent value is expected to peak, indicating a more viscous response from the film. Within the temperature range that we tested in this study, we were unable to reach T_g of the nanocomposites. Pure chitosan film has T_g above 160 °C [46,47]. Because gold nanoparticles possess attractive interaction to pure chitosan matrix, we expect the T_g of the Au–chitosan nanocomposites to be increased relative to that of pure chitosan as a result of a strong interfacial interactions. These results indicate that the addition of gold nanoparticles to pure chitosan significantly alters its viscoelastic response. Gold nanoparticles are 3D nanostructures, due to a strong interfacial interaction between gold nanoparticles and chitosan, the chitosan–Au nanocomposites are expected to show enhanced properties. The enhancement effects is related to the dispersion state of nanoparticle in the chitosan matrix and their interfacial interactions [48]. The mechanical properties enhancement could be attributed to the interaction between gold nanoparticles and chitosan as is confirmed by the high value of gold/chitosan nanoparticles' Zeta-potential (above 62 mV) and also the uniform distribution of gold nanoparticles in the chitosan matrix (see Table 1). The reduced moduli of all the gold reinforced chitosan nanocomposite films tested were significantly higher than that of pure chitosan film. Similar enhancement effects have been reported in the literature. Jayasuriya et al. [49] observed positive effects on nano- and micro-hardness as percentage of ZnO nanoparticles was increased in chitosan matrix. Garcia et al. [28] obtained more elastic and less hard zein–chitosan composite films as zein concentration was increased in the material. In a study of structural and mechanical properties of halloysite–chitosan bionanocomposites for tissue engineering, Liu et al. [46] found improved tensile and viscoelastic properties as halloysite concentration is increased. From a study of chitosan–nanohydroxyapatite (nHA)

nanocomposites for bone tissue engineering, Maganti et al. [50] reported the positive effect of nHA concentration on mechanical properties without affecting other essential properties exhibited by the pure chitosan films. Graphane oxide–chitosan nanocomposites developed for bio- and electro-chemical applications showed a significant increase in tensile strength and Young's modulus upon introducing minute amount of graphane oxide reinforcement [21,26]. The positive effects of gold nanoparticle reinforcement on both mechanical stability and cellular response were observed while gold nanoparticle reinforced chitosan nanocomposites were studied as a material used for peripheral nerve regeneration [47]. Wang et al. [25] achieved significant improvement in indentation hardness, tensile modulus and tensile strength of chitosan film upon reinforcement with carbon nanotubes.

3.3. Cytocompatibility analysis of Au–chitosan bio-nanocomposite films

For these composite films to find applications in the biomedical field, they must be non-toxic and biocompatible. The cytocompatibility of Au–chitosan nanocomposites was tested *in vitro* by analyzing cell proliferation. To determine the appropriate gold concentration in the chitosan matrix, we utilized *in vitro* human epithelial cells derived from cervix tissue. The biological properties in different Au–chitosan nanocomposites and the behavior of human epithelial cells on the biomaterials were compared. Given that the pure chitosan microstructure is altered by the inclusion of gold nanoparticles, it is expected that the nanoparticles could also modulate the cellular response. The viability of the human epithelial cells exposed to chitosan and chitosan–Au nanocomposites with different gold concentrations ranging from 0 mg/ml–0.25 mg/ml is summarized in Fig. 12. The results reveal that human epithelial cells can adhere and proliferate on both pure chitosan and Au–chitosan nanocomposites. The cells seeded on chitosan and Au–chitosan nanocomposites were clearly observed using a phase contrast microscope (Fig. 11). The rate of cell proliferation was dependent on the concentration of gold in the chitosan matrix. At day 1 culture, there was no significant difference between the samples. With increased culture time (day 3 and day 5), more cells were present on Au–chitosan reinforced nanocomposites compared to pure chitosan ($P < 0.05$).

The morphology of these cells seeded on the films showed that they were viable and dividing. Our results show that adding a certain amount of gold nanoparticles stimulates cell proliferation. This could be attributed to an increase in the chitosan film surface area available for cells to adhere and proliferate. However, there is a threshold amount of gold amount (0.20 mg/ml in our case) above which additional amount of gold nanoparticles used results in a decrease in cell proliferation. These results are consistent with previously published work [47,51] that have also reported a decreasing proliferation effect with higher amount of gold concentration in a polymer nanocomposite.

4. Conclusions

Gold nanoparticle reinforced chitosan nanocomposite films were prepared via solution evaporation technique. The composite solutions were characterized using SEM, TEM, UV–vis and Zeta potential for the gold particle dispersion, entrapment efficiency and aggregation of gold nanoparticles in the chitosan matrix. Uniform distribution of gold nanoparticle was

achieved throughout the chitosan matrix. The nanomechanical properties of both pure chitosan and Au–chitosan bio-nanocomposites were characterized by nanoindentation techniques. The obtained nanocomposite films were confirmed to be biocompatible by their ability to support growth of human cells *in vitro*. The results show that addition of gold nanoparticles to pure chitosan not only enhanced the mechanical properties but also had influence on cellular response and biocompatibility of the Au–chitosan bio-nanocomposites. In summary, the results show enhanced mechanical properties with increase in gold nanoparticle concentration and provide better understanding of structure–property relationship of such biocompatible materials for potential biomedical applications. The results obtained demonstrate the possibility to modulate the mechanical response of these multifunctional materials taking into account the final application.

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Appendix 1

Nanoindentation has made great advances in the studies of nanomechanics. The technique relies on the local deformation induced on a material's surface with an indenter of known properties under the application of a given load. The load applied on the indenter and the corresponding displacement into a sample is continuously monitored during the loading and unloading processes. The attractiveness of nanoindentation is its ability to characterize the mechanical behavior of small volumes of material with spatial resolutions in the nanometer to micrometer range. In quasi-static nanoindentation experiments, the Oliver–Pharr method [52] is used to extract the indentation modulus and hardness from the force-displacement

curves. The indentation modulus (E_i) is calculated from the unloading portion of the force–displacement curve according to the relation

$$E_i = \left[2 \sqrt{\frac{A_c}{\pi}} \frac{1}{S} - \frac{1-\nu_t^2}{E_t} \right]^{-1} = \frac{E_s}{1-\nu_s^2}, \quad (\text{A1})$$

where A_c is the area of contact and S is the contact stiffness. E and ν are the Young's modulus and Poisson's ratio, respectively, where the subscripts correspond to the sample (s) and diamond indenter tip (t) elastic properties. The hardness (H) is the ratio of the maximum force to the contact area, or

$$H = \frac{P_{max}}{A_c}. \quad (\text{A2})$$

For a spherical tip of radius (R), contact area is given by, $A_c = \pi h_c^2 + 2\pi R h_c$, here h_c is the contact depth.

During dynamic nanoindentation, the instrument allows a small sinusoidal load to be superimposed onto the overall quasi-static load. The displacement response is measured at the same frequency as the applied oscillating load and the resulting phase lag is related to the material damping. The sample stiffness K_s and damping C_s can be expressed in terms of indenter parameters as: [53]

$$K_s(\omega) = \left| \frac{F_0}{z_0} \right| \cos(\delta) + m\omega^2 - K_i, \quad \text{and} \quad C_s(\omega) = \left| \frac{F_0}{z_0} \right| \frac{\sin(\delta)}{\omega} - C_i \quad (\text{A3})$$

where, F_0 is the force amplitude, m is the indenter mass, z_0 is the displacement amplitude, ω is the excitation frequency, and δ is the phase angle associated with damping between the applied force and the resultant displacement. The indenter stiffness and damping are represented by K_i and C_i respectively. By making use of Hertzian [45] contact mechanics, the storage indentation modulus (M') is expressed as,

$$M' = (k_{storage}/2) \sqrt{\pi/A_c}, \quad (\text{A4})$$

and the loss indentation modulus (M'') is given by

$$M'' = (k_{\text{loss}}/2) \sqrt{\pi/A_c}, \quad (\text{A5})$$

here, ν is Poisson's ratio, A_c is the contact area between the tip and sample. A useful measure of the relative contributions of the storage and loss moduli to the mechanical response of a material is given by the loss factor defined as,

$$\tan(\delta) = k_{\text{loss}}/k_{\text{storage}}, \quad (\text{A6})$$

which is a measure of damping in a linear viscoelastic material.

Fig. 1. UV-vis spectra of (a) Pure Au nanoparticles vs chitosan-Au nanocomposites and (b) chitosan-Au nanocomposites at different concentration of Au particles.

Fig. 2. (a) TEM micrograph of chitosan–Au nanocomposites, (b) size distribution histogram of chitosan–Au nanocomposites in aqueous solution and (c) selected area electron diffraction (SAED) chitosan stabilized gold nanoparticles.

Fig. 3.

Cartoon representation of participating functionality in Au nanoparticle formation in chitosan matrix.

Fig. 4.
X-ray diffraction of chitosan-Au nanocomposites.

Fig. 5.

FE-SEM images of pure chitosan and chitosan–Au composites at different Au-NP concentration. The scale bar corresponds to 100 nm in the SEM images.

Fig. 6.
EDX analysis of gold chitosan nanocomposite.

Fig. 7.
Representative load vs displacement curves of gold/chitosan composites with different concentrations of gold.

Fig. 8.

(a) Reduced modulus vs gold concentration at different test temperatures. Reduced modulus increases with increasing gold concentration, while an increase in temperature results in lower reduced modulus values. (b) Hardness vs gold concentration at different test temperatures.

Fig. 9. Plots of (a) storage modulus, and (b) loss tangent versus frequency at different test temperatures.

Fig. 10.

(a) Storage modulus, and (b) loss tangent versus frequency at different test temperatures.

Fig. 11.

Phase contrast microscopy images of the nanocomposite films after day 1, day 3 and day 5.

Fig. 12. Results of cell growth on different composite films. The bar represents the standard deviation.

Table 1

Zeta potential of chitosan–Au composite synthesized using varying concentrations of gold nanoparticles.

Au concentration in chitosan solution (mg/ml)	0.10	0.15	0.20	0.25
Zeta potential (mv)	+66.87	+70.09	+62.74	+62.99

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Table 2
The ratio between the intensities of the { 1 1 1 } and { 200 } reflections of different chitosan–Au composites.

Au concentration in film (mg/ml)	0.00	0.10	0.15	0.20	0.25
Intensity ratio { 111 }/{ 200 }	0.00	1.32	1.39	2.17	2.80