Improvement in Water Stability and other related Functional Properties of thin Cast Kafirin Protein Films

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ABSTRACT

Improvement in the water stability and other related functional properties of thin (<50 μm) kafirin protein films was investigated. Thin conventional kafirin films and kafirinmicroparticle films were prepared by casting in acetic acid solution. Thin kafirin films cast from microparticles were more stable in water than conventional cast kafirin films. Treatment of kafirinmicroparticles with heat and transglutaminase resulted in slightly thicker films with reduced tensile strength. In contrast,glutaraldehyde treatment resulted in up to 43% increase in film tensile strength. The films prepared from microparticles treated with glutaraldehyde were quite stable in ambient temperature water, despite the loss of plasticizer. This was probably due to the formation of covalent cross-linking between free amino groups of the kafirin polypeptides and carbonyl groups of the aldehyde. Thus, such thin glutaraldehyde-treated kafirinmicroparticle films appear to have good potential for use as biomaterials in aqueous applications.

KEYWORDS: Kafrinmicroparticles; thin cast kafirin films; transglutaminase; glutaraldehyde; water stability; tensile properties

INTRODUCTION

Films and other biomaterials made from proteins draw much interest as a consumer- and environment-friendly option to synthetic polymer products (*1*). Applications or potential uses of protein biomaterials include packaging materials and food coatings (*2*), carriers of antimicrobial agents (*3*) and drug-eluting coating film for cardiovascular devices (*4*). However, with respect to films made from cereal proteins, their poor mechanical properties and water stability compared to similar materials made from synthetic polymers are major limitations (*5*).

Protein film functional properties, including water stability, can be modified by physical treatments such as heat (*6*), γ-irradiation (*7*), chemicals such as aldehydes (*8*) or enzymes such as transglutaminase (*9*). Most of these modifications have been done on films with >50 μm thickness. For example, Sessa et al. (*8*) worked on 700 to 900 μm thick zein films, Chambi and Grosso (*9*) worked on 750 μm thick gelatin and casein films, while Hernández-Muñoz et al. study (*10*) was on 55 μm thick gliadin films.

Kafirin, the sorghum prolamin protein, is relatively hydrophobic compared to other cereal proteins such as zein (*11*). Thus, kafirin has potential for use in biomaterials that require stability in water. Our research has shown that thin cast films (<50 μm) produced from kafirinmicroparticles have some superior functional properties such as smoother film surface and lower water vapor permeability, than the conventional cast films from kafirin (*12*). With regard to protein microparticles, studies have focused on treatments to improve their heat stability, microencapsulation efficiency and release profiles (*13*-*15*) but not to enhance functional properties of films prepared from them. This study investigated various treatments to improve the water stability and other related functional properties of thin kafirin films.

MATERIALS AND METHODS

Materials

Kafirin was extracted from mixture of two very similarwhite, tan-plant non-tannin sorghum cultivars PANNAR PEX 202/606, as described (*16*).Briefly, whole milled sorghum was mixed with 70% (w/w) aqueous ethanol containing 3.5% (w/w) sodium metabisulfiteand 5% (w/w) sodium hydroxide and then heated at 70°C for 1 h with vigorous stirring. The clear supernatant was recovered by centrifugation and the ethanol evaporated. Kafirin was precipitated by adjusting the pH of the protein suspension to 5.0. The precipitated kafirin was recovered by vacuum filtration, freeze-drying, defatting with hexane at ambient temperature and air-drying. All the chemicals were obtained from Merck, Darmstadt, Germany or Sigma, St. Louis, MO, unless otherwise stated.

Preparation of Conventional Cast Kafirin Films

Conventional kafirin films were prepared as by the casting method described (*17*) from a 2% (w/wprotein basis) kafirin solution in glacial acetic acid, containing 40% (w/w with respect to protein) plasticizer (a 1:1:1 w/w mixture of glycerol: polyethylene glycol 400: lactic acid).We have previously shown this plasticizer combination to be effective in kafirin films. (*12*)Films were dried overnight at 50°C in an oven (not forced draft) on a level surface.

Preparation of KafirinMicroparticle Films

To prepare kafirinmicroparticle films, firstly, kafirinmicroparticles were prepared using acetic acid solvent according to Taylor et al. (*12*) with some modification.Plasticizer (0.66 g) was mixed with glacial acetic acid (4.34 g) and added to kafirin (1.9 g, 84% protein) with gentle stirring until fully dissolved. The kafirin solution held at ambient temperature (22°C)

for 16 h to equilibrate. Then,kafirinmicroparticles were prepared by adding 73.1 g distilled water to 6.9 g kafirin solution at a rate of 1.4 mL/min using a Watson-Marlow Bredel peristaltic pump (Falmouth, U.K.), while mixing using a magnetic stirrer at 600 rpm at ambient temperature. This suspension contained 2% (w/w) kafirin protein and 5.4% (w/w) (0.9 M) acetic acid.

To cast the films, 4 g suspensions of kafirinmicroparticleswere centrifuged at 3150 *g* for 10 min. The clear supernatants were carefully removed and replaced by 25% (w/w) (4.2 M) acetic acid and then left to equilibrate at ambient temperature for 12 h. Then, 32 mg plasticizer (40% w/w on kafirin protein basis) was added and films cast in the bottom dishes (90 mm diameter) of 100 mm × 15 mm borosilicate glass Petri dishes (Schott Glas, Mainz, Germany) by drying overnight at 50°C in an oven (not forced draught). All the films were assessed visually and photographed using a flatbed scanner.

Preparation of Films from Heat-treated KafirinMicroparticles

Kafirinmicroparticle film preparation suspension (4 g), described above, was washed with distilled water three times to remove the acetic acid. Treatment of kafirinmicroparticles with heat was done by heating the microparticles suspended in water, at 50°C, 75°C and 100°C for 1 h. A control film preparation mixture was maintained at ambient temperature for the same period. Then, supernatants were replaced with 25% acetic acid, plasticizer added and films cast as described.

Preparation of Films from Transgutaminase-treated KafirinMicroparticles

The supernatants in the 4 gkafirinmicroparticle suspensions were removed by centrifugation and washed with distilled water as described above. The supernatants were replaced with 0.1%, 0.3% and 0.6% (w/w) microbial transglutaminase (ActivaWM), activity 100 U/g

(Ajinomoto Foods Europe, S.A.S., France) (on protein basis) in 0.02 MTris–HCl buffer (pH 7.0). The buffer had been pre-heated to 50°C prior to dissolving the enzyme. For control films, 25% pure maltodextrin in 0.02 MTris–HCl buffer, pH 7.0 was used as the transglutaminase enzyme is supplied in a 99% maltodextrin carrier base. Transglutaminase reaction was carried out at 30°Cfor 12 h. Optimal transglutaminase reaction temperature is 50°C (*18*)but complete films could not be formed if this temperature was used, probably because of heat induced cross-linking of the kafirin proteins. The films were cast as described.

Preparation of Films from Glutaraldehyde-treated KafirinMicroparticles

These were prepared by adding 10%, 20% and 30% (w/w) glutaraldehyde (Saarchem, Krugersdorp, South Africa) (as a proportion of protein) to 4 g film preparation mixtures in 25% acetic acid (pH 2.0). Reaction with glutaraldehyde was carried out at ambient temperature for 12 h and the films cast as described.

Analyses

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Films were characterized by SDS–PAGE under reducing and non-reducing conditions. An XCellSureLock™ Mini-Cell electrophoresis unit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used with 15-well 1 mm thick pre-prepared Invitrogen NuPAGE® 4–12% Bis-Tris gradient gels. The loading was ≈10 μg protein. Invitrogen Mark12™ Unstained Standard was used. Proteins were stained with Coomassie® Brilliant Blue R250 overnight, destained and photographed.

Scanning Electron Microscopy

The film surfaces were examined using Scanning Electron Microscopy (SEM) as described (*12*) with a Jeol JSM-840 Scanning Electron Microscope (Tokyo, Japan).

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy was performed as described (*12*). Samples were scanned in a Vertex 70v FT-IR spectrophotometer (BrukerOptik, Ettlingen, Germany), using 64 scans, 8 cm^{-1} band and an interval of 1 cm in the Attenuated Total Reflectance (ATR) mode. The FTIR spectra were Fourier-deconvoluted with a resolution enhancement factor of 2 and a bandwidth of 12 cm^{-1}.

Tensile Properties

The films were conditioned at 50% relative humidity and 25°C for 72 h in a desiccator maintained using 4.99 M calcium chloride (*19*). Tensile properties of the films were studied as described (*17*), based on ASTM D882–97 method (*20*), using a TA-XT2 Texture Analyser (Stable Micro Systems, Goldalming, U.K.). For soaked films, the films were air-dried at ambient temperature for 30 min before measuring tensile properties.

In vitro Protein Digestibility

This was performed based on the pepsin digestion procedure of Hamaker et al. (*21*). Prior to *in vitro* protein digestibility (IVPD) assay, the films were freeze-fractured in liquid nitrogen and ground into powder using a mortar and pestle. P7000-100G pepsin, activity 863 units/mg protein, was used.

Surface Density

Film surface density was measured based on the procedure of Soliman et al. (*7*). Films (90 mm diameter) were weighed. The weight of each film was divided by its area to calculate the surface density (mg/cm^2).

Water Stability

Complete conventional cast kafirin films and kafirinmicroparticle films were immersed in water (22^oC) containing 0.02% (w/v) sodium azide in separate transparent plastic containers then mounted on an orbital shaker set at a gentle speed of 70 rpm and monitored over a period of 48 h. To determine the extent to which the films would maintain their physical integrity in water under vigorous shaking, complete films were immersed in distilled water containing sodium azide at 22°C, and then mounted on an orbital shaker set at speed of 300 rpm for 72 h. At the end of the shaking period, the films were visually assessed and photographed using a flatbed scanner in the plastic containers.

Water Uptake and Weight Loss of Films in Water

These were measured according to Soliman et al. (*7*) with some modification. Films (90 mm diameter) were dried in a desiccator for 72 h and then weighed. The dried films were immersed in distilled water for 24 h at 22^oC with gentle agitation on a rocking platform set at 30 rpm. The water on the film surfaces was removed by placing the films in between a paper towel and then weighed. The films were then dried ina desiccator as before and weighed again. The film water uptake and weight loss in water were calculated thus:

$$\%\ \text{Water uptake} = \frac{(\text{Mass after immersion} - \text{Initial dry mass})}{\text{Initial dry mass}} \times 100$$

$$\%\ \text{Weight loss in water} = \frac{(\text{Initial dry mass} - \text{Mass after immersion and drying})}{\text{Mass after immersion and drying}} \times 100$$

Statistical Analyses

All experiments were repeated at least twice. Data were analyzed by one-way analysis of variance (ANOVA). Significant differences among the means were determined by Fischer's least significant difference (LSD) test.

RESULTS AND DISCUSSION

Water Stability of Conventional Cast Kafirin Films and Cast KafirinMicroparticle Films

As shown (**Figure 1)** conventional cast kafirin films disintegrated into many small fragments in water, while the cast kafirinmicroparticle films remained largely intact. The better stability of kafirinmicroparticle films in water is probably a result of better film matrix cohesion due to better solubilization of kafirin in the film casting solvent. This can be attributed to the large voids within the kafirinmicroparticles, when prepared by coacervation.(*12*) Because cast kafirinmicroparticle films had better water stability than cast kafirin films, various cross-linking treatments were applied to the former to further enhance their functional properties.

Film Physical Appearance

Treating kafirinmicroparticles with heat at moderate temperature (50^{o}C) did not change the clarity of the films made from them compared to control(**Figure 2A- a,b**). However, SEM revealed that the film surfaces were rough (**Figure 2A- e, f**). Higher temperature treatment (75^{o}C and 100^{o}C) resulted in the formation of opaque and incomplete films (arrows, **Figure 2A- c, d**). These films had a rough surface and the microparticles were poorly fused (arrows, **Figure 2A-g, h**), which was similar to the appearance of biomaterials made from zein microparticles such as films (*22-23*) and sponges (*24*). Previous work has shown that films

prepared from microwave-heated kafirin had a rough surface, which was attributed to undissolved lumps of kafirin (*6*). The poor fusion of the heated kafirinmicroparticles was probably due to reduction in their solubility in the aqueous acetic acid casting solution.Hamaker et al. (*21*) suggested that the reduction in solubility of kafirin proteins as a result of thermal treatment is probably due to disulfide cross-linking of kafirin proteins.

Transglutaminase-treated kafirin microparticle films were as clear as their control (**Figure 2B, i–l**) but had a rough surface when viewed by SEM (**Figure2B, m–p**), indicating poor fusion of the microparticles. Tang et al. (*25*) also reported a rough and uneven surface of films prepared from transglutaminase-treated soy protein isolate.In this present study, Maillard reaction due to the presence of maltodextrin, may have caused poor solubility of the microparticles, thereby resulting in an uneven film surface.

Treatingkafirinmicroparticles with glutaraldehyde did not change the clarity of the films irrespective of the concentration of glutaraldehyde (**Figure 2C, q–t**). However, the glutaraldehyde-treated kafirinmicroparticle films were slightly rough (**Figure 2C, v–x**),probably due to reduction in solubility of the kafirinmicroparticles as a result of glutaraldehyde cross-linking of the kafirin proteins. However, the glutaraldehyde-treatedkafirinmicroparticles were clearly better fused than the heat-treated microparticles.

Film Chemical Structure

SDS–PAGE under non-reducing and reducing conditions of films prepared from heat-treated kafirinmicroparticles and untreated control had highest band intensity at 16–28 kDa (**Figure 3A**), which are the monomeric α-, β- and γ-kafirins (*26*). Bands of 42–58 kDa and 61–88 kDa, identified as dimers and trimers, respectively (*27*), were present in all the films, indicative of kafirin polymerization. Treating kafirinmicroparticles with heat did not result in

any change in band pattern with SDS–PAGE under non-reducing conditions. Any effect of low temperature treatment of microparticles was probably masked by the subsequent drying temperature of 50^{o}C for 12 h which all the films underwent. With heat treatment at 100^{o}C some oligomers (≈94–183 kDa) (arrow in **Figure 3A**, lane 4 under reducing conditions) resistant to reduction by mercaptoethanol remained. These may be attributed to inaccessible disulfidecross-links and/or non-disulfide inter-polypeptide cross-links formed by oxidative coupling of tyrosine residues induced by heat as suggested by Duodu et al. (*28*)

Despite changes in the physical appearance of films, SDS–PAGE showed no evidence of transglutaminase-induced kafirin polymerization (**Figure 3B**).This was probably because kafirin has very low lysine content (*11*), which renders it a poor substrate for the transglutaminase reaction that produces ε-(γ-glutamyl)-lysine bridges. For a lysine-poor protein, transglutaminase-catalyzed protein reaction may progress through deamidation, which is an alternative reaction pathway (*29*). The results in the present study are consistent with those of Motoki et al. (*30*) working on glutamine-specific deamidation of α_{s1}-casein by transglutaminase who found no changes in molecular weights. Similarly, Flores et al. (*31*) working on maize protein isolate emulsions found similar electrophoretic band pattern for deamidated and non-deamidated emulsions, despite their difference in emulsion stability. The fact that films prepared from transglutaminase-treated kafirinmicroparticles were rough (**Figure2B, n–p**) may be attributed to an increase in negative charges in the kafirin due to deamidation, making the microparticles less soluble in acetic acid casting solution.

Glutaraldehyde treatment of the kafirinmicroparticles resulted in the reduction in monomer (17–28 kDa), dimer (45–61 kDa), trimer (66–83 kDa) and oligomer (>94 kDa) band intensities with the SDS–PAGE under non-reducing conditions (**Figure 3C**). With SDS–PAGE under reducing conditions, there was an increase in intensities ofdimer,trimer and

oligomer bands for 10% glutaraldehyde treatment (**Figure 3C**, lane 2), indicating the presence of polymerized kafirin proteins. The presence of polymerized kafirin under reducing conditions was expected as cross-links with glutaraldehyde are formed between free amino groups of peptide chains and the carbonyl groups of the aldehyde (*32*) and not disulfide linkages. Treatments with higher glutaraldehyde concentrations (20% and 30%) resulted inthe monomer, dimer and trimer bands being much fainter and an almost complete absence of oligomer bands. These observations indicate formation of highly polymerized kafirin polymersof MW > 200 kDaat higher glutaraldehyde concentrations, which were too large to migrate into the separating gel. A similar result was reported by Reddy et al. (*5*) who found disappearance of molecular weight bands, when they cross-linked pre-formed wheat gluten fibers with high concentration of glutaraldehyde.

FTIR

Treating the kafirinmicroparticles with heat decreased the relative proportion of α-helical conformation in the films by up to 11%, at the amide I band (wave number ≈1650–1620 cm^{-1}) (**Table1**). There was a decrease in the relative proportion of α-helical conformation with increase in heating temperature. Byaruhanga et al. (*33*) reported a similar decrease in proportion of α-helical conformation with concomitant increase in β-sheet conformation when they heated kafirin and kafirin film with microwave energy. Similarly, Emmambux and Taylor (*34*) reported a reduction in α-helical conformation when they studied the protein secondary structure in cooked kafirin. It has been suggested that during thermal treatment the hydrogen bonds stabilizing protein structure are disrupted, causing loss of the α-helical and β-sheet conformation and creating new β-sheet arrangements (*35*).Loss of α-helical conformation can be indicative of protein aggregation (*36*). In the present study, the relative proportion of α-helical conformation in the amide I band for control films was 53.9%,

slightly higher than 50.5% found by Taylor et al. (*12*) for similar kafirinmicroparticle films. Such differences have been attributed to the kafirin batch variations (*12*).

Transglutaminase treatment of kafirinmicroparticles caused up to 5% reduction in the relative proportion of α-helical conformation with respect to control films with maltodextrin treatment. As with increasing level of heat treatment, there was a progressive decrease in the relative proportion of α-helical conformation with increase in concentration of transglutaminase. This was probably due to transglutaminase-induced deamidation of kafirin proteins causing increased electrostatic repulsion and the decreased hydrogen bonding (*37*). These findings are similar to those from soymilk protein residue (*38*) and gluten (*39*).

In contrast to the trend with heat and transglutaminase treatments, there was a progressive increase in the relative proportion of α-helical conformation with increase in glutaraldehyde concentration. Selling et al. (*40*) reported similar increase in α-helical conformation of zein fibers treated with glutaraldehyde. As with transglutaminase treatment, the changes in the protein secondary structure due to treatment with glutaraldehyde were generally small compared to heat treatment. This is consistent with findings by Caillard et al. (*41*) on soy protein hydrogels cross-linked using glutaraldehyde, which showed changes in gel physical appearance and functional properties that were not reflected to the same extent by alterations in the protein secondary structure.

Film Functional Properties

Film Water Stability, Water Uptake and Weight Loss in Water

Transglutaminase treatment resulted in poor water stability of the kafirinmicroparticle films (**Figure4A**), probably due to transglutaminase-induced deamidation resulting in the poor fusion of the kafirinmicroparticles (**Figure2B**).

In contrast, treating kafirinmicroparticles with glutaraldehyde resulted in films that were resistant to disintegration in water (**Figure4B**). SEM of the films after soaking in water for 72 h with vigorous agitation (**Figure5**) showed the presence of fewer holes (arrows in **Figure5**) on the films prepared from glutaraldehyde-treated kafirinmicroparticles compared to untreated control, indicating that less physical damage occurred. There was an increase in film stability in water with increasing glutaraldehyde concentration. Increase in water stability has been reported when glutaraldehyde is used to modify a number of similar protein biomaterials such as zein films (*8*), gliadin films (*10*), zein fibers (*40*) and gluten fibers (*5*). Glutaraldehyde treatment resulted in reduction of both film water uptake and film weight loss in water (**Table 2**). Similar reductions in film water uptake and weight loss in water as result of glutaraldehyde treatment has been reported by Orliac et al. (*42*) working on sunflower protein isolate films. Likewise, glutaraldehyde treatment has been shown to cause reductions in weight loss in water with gliadin films (*10*). The film weight loss in water was evidently due mainly to plasticizer loss as the reduction in film weight was equivalent to 88–95% of the plasticizer content. This was also reported by Hernández-Muñoz et al. (*10*). The reduction in film water uptake or weight loss in water as a result of glutaraldehyde treatment is probably due to the covalent bonding leading to the formation of a more stable cross-linked network resistant to water. Reddy et al. (*5*) proposed two reasons for improvement in water stability after treatment with glutaraldehyde. First, treatment with glutaraldehyde may result in fewer hydrophilic groups, when the aldehydes react with free amino groups of the basic amino acids, which are among the primary water-bonding sites on proteins. Second, the formation of higher molecular weight proteins by glutaraldehyde treatment may result in better water resistance than lower molecular weight proteins in the control. This latter explanation concurs with SDS–PAGE data (**Figure 3C**), which showed presence of polymerized kafirins in glutaraldehyde-treated kafirinmicroparticle films.

As shown (**Figure2A**), treating kafirin microparticles with heat resulted in formation of incomplete films, especially when subjected to higher temperature treatments. Therefore, no data were obtained forwater stability, water uptake and weight loss in water for heat cross-linked films. Also, as transglutaminase treatment resulted in poor water stability of the films (**Figure 4A**), the determination of water uptake and weight loss in water was not feasible for these films.

Film Tensile Properties Before and After Soaking

The control kafirin microparticle films were approx. 16 µm thick (**Table 3**), similar to the 14 µm reported previously *(12)*. Heat treatment of kafirinmicroparticles resulted in filmsthat were 20–29% thicker. With regard to film tensile properties before soaking, there was a progressive reduction in maximum stress with increase in treatment temperature. The increase in film thickness and the reduction in film maximum stress was probably because, as noted by SEM (**Figure 2A**), these films had poorly fused microparticles, indicating poor structural cohesion of film matrix. As discussed, treating kafirinmicroparticles with heat resulted in formation of incomplete films, especially the higher temperature treatments. Therefore, no tensile data were obtained forthe soaked heat-treated films.

Transglutaminase treatment resulted in thicker films and a 24–53% reduction in film maximum stress, with respect to control films with maltodextrin treatment. As with heat treatment, the poor fusion of the microparticles due to treatment with transglutaminase (**Figure 2B**) could probably be a reason for the increase in thickness and weakness of these films. In addition, transglutaminase-induced deamidation may have been a contributing factor. As discussed, the transglutaminase-treated films disintegrated during water stability test rendering it impractical to obtain data for tensile properties after soaking the films.

Surface density was determined for glutaraldehyde-treated kafirinmicroparticle films as SDS–PAGE showed evidence of cross-linking (**Figure 3C**). Glutaraldehyde treatment slightly increased the film surface density, while it resulted in varied effects on film tensile strength, depending on concentration of glutaraldehyde (**Table 3**). With 10% glutaraldehyde treatment, there was a 43% increase in film maximum tensile stress, and 58% increase in maximum strain. However, at higher glutaraldehyde concentration (20% and 30%), there was a 42–43% reduction in film breaking stress accompanied by about four-fold increase in maximum strain. Increase in the film maximum stress as a result of glutaraldehyde treatment is presumably due to formation of additional cross-links, as discussed. Similar increase in protein film tensile strength has been reported when glutaraldehyde is used to cross-link many proteins such as gliadin (*10*), zein (*8*, *43*) and sunflower protein isolate (*42*). On the other hand, the fact that there was a reduction in maximum stress accompanied by increase in maximum strain at higher glutaraldehyde concentration was probably due to plasticizer effect of excess glutaraldehyde molecules in the protein network. Similar results were reported by Marquié et al. (*44*) working on cottonseed protein films who noted a decrease in film maximum puncture force. Likewise, increase in elongation at break has been reported for different types of protein films cross-linked using glutaraldehyde such as zein films (*43*).Marquié et al. (*44*) proposed that molecular structure of the long bridges formed between the glutaraldehyde cross-linked proteins may lead to a decrease in the intermolecular forces between polymer chains.

Concerning tensile properties of films that had been soaked for 24 h in water, glutaraldehyde treatment resulted in retention of far higher tensile strength and strain than the control (**Table 3**). These results agree with the data on film water stability (**Figures 4B** and **5)**, water uptake and weight loss in water (**Table 2**), which showed that glutaraldehyde treatment resulted in kafirinmicroparticle films with better integrity even after soaking in water. Soaked film

tensile strength retention seems to be similar to published glutaraldehyde treatment data, despite the thinness of the films. For example, tensile strength of soaked zein films was ≈31% of the pre-soaked film tensile strength with 8% glutaraldehyde treatment for films of 700 to 900 μmthickness (*8*), similar to the 34% obtained with 10% glutaraldehyde treatment in the present study. The reduction in the tensile strain of the kafirinmicroparticle films after soaking in water was probably due to the washing out of the plasticizer.

Filmin Vitro Protein Digestibility

Heat treatment of kafirinmicroparticles slightly reduced the *in vitro* protein digestibility (IVPD) of the films made from them (**Table 2**). Byaruhanga et al. (*6*) reported a reduction in IVPD of cast kafrin films as a result of heat treatment. Reduction in IVPD of wet heated-kafirin is a known phenomenon, which is attributed to disulfide cross-linking (*28*). However, in the present study, the rather small IVPD reduction was probably due to the prolonged heat treatment of all the films, as discussed. As with heat treatment, treating kafirinmicroparticles with transglutaminase reduced film IVPD. A reduction in protein digestibility has been reported for soy protein isolate treated with transglutaminase (*45*). The film IVPD was lower with transglutaminase treatment than with heat treatment. This is probably due to the fact that maltodextrin (a carbohydrate), which is the transglutaminase enzyme carrier base constituting 99% of the enzyme material, may have interacted with kafirin through Maillard reaction thereby reducing further IVPD. Maillard reaction was indicated by the fact that the treated films had a slight yellowish tinge (data not shown).

In contrast, microparticle treatment with glutaraldehyde increased the kafirinmicroparticle films IVPD by up to some 12%. The increase in protein digestibility as a result of glutaraldehyde treatment may probably be due to an increase in free volume. Migneault et al. (*32*) suggested that it is polymeric forms of glutaraldehyde (polyglutaraldehydes) that are

involved in the cross-linking of proteins. These glutaraldehyde polymers may form long distance methylene bridges between protein chains, probably creating easier access of the pepsin enzymeto the peptide bonds in the kafirin.

Overall, the changes in IVPD of the kafirinmicroparticle films as a result of all the three treatments were not large (maximum of 20% change) probably due to the exposure to relatively high temperature during solvent evaporation.

In conclusion, thin kafirin films cast from microparticles are more stable in water than conventional cast kafirin films. Glutaraldehyde treatment of the kafirinmicroparticles renders the films quite stable in ambient temperature water, despite the loss of plasticizer. Thus, such thin (<50 μm) glutaraldehyde-treated kafirinmicroparticle films appear to have good potential for use as biomaterials in aqueous applications.

ABBREVIATIONS USED

FTIR, Fourier transform infrared spectroscopy; IVPD, *in vitro* protein digestibility; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, scanning electron microscopy.

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FIGURE CAPTIONS

Figure 1. Appearance of kafirin films cast in acetic acid, after immersion in water for 48 h at ambient temperature with gentle shaking (70 rpm). Films photographed in a transparent plastic tub while immersed in water: **(A)**. Conventional kafirin film. Arrows point to film fragments. **(B).**Kafirinmicroparticle film. Arrow points to torn section of film.

Figure 2. Physical appearance of films prepared from treated kafirinmicroparticles: **(A)** Heat treatment. **a-d**: photographs, **e-h**: SEM. **a**, **e** - control; **b**, **f** - 50^{o}C; **c**, **g** - 75^{o}C; **d**, **h** - 100^{o}C. Arrows: **c**, **d**- opaque incomplete sections; **g**, **h** –gaps. **(B)**Transglutaminase (TG) treatment.**i-l**: photographs; **m-p**: SEM. **i**, **m** - Maltodextrin; **j**, **n** - 0.1% TG + Maltodextrin; **k**, **o** - 0.3% + Maltodextrin; **l**, **p** - 0.6% TG + Maltodextrin. **(C)**Glutaraldehyde treatment. **q-t**: photographs, **u-x**: SEM. **q**, **u** - control; **r**, **v** - 10%; **s**, **w** - 20%; **t**, **x** - 30%.

Figure 3. SDS–PAGE of films prepared from treated kafirinmicroparticles. Protein loading, 10 µg:

(**A)**. Heat treatment. Lanes M: Molecular markers; 1: Control (22ºC); 2: 50ºC; 3: 75ºC; 4: 100^{o}C. Arrow: reduction-resistant oligomers.

(B). Transglutaminase (TG) treatment. Lanes M: Molecular markers; 1: Maltodextrin; 2: 0.1% TG + Maltodextrin; 3: 0.3% TG + Maltodextrin; 4: 0.6% TG + Maltodextrin.

(C). Glutaraldehyde treatment. Lanes M: Molecular markers; 1: Control; 2: 10%; 3: 20%; 4: 30%.

Figure 4. Water stability of films prepared from treated kafirinmicroparticles. The films were photographed in Petri dishes in water: **(A)**. Transglutaminase (TG) treatment (**a-d**). **a**: Maltodextrin; **b**: 0.1% TG + Maltodextrin; **c**: 0.3% TG + Maltodextrin; **d**: 0.6% TG + Maltodextrin. Arrows: film fragments. **(B)**. Glutaraldehyde treatment (**e-h**). **e**: Control; **f**: 10%; **g**: 20%; **h**: 30%; Arrows- **e**: fragmented film; **f**, **g**: disintegrated section of film. Heat treatment resulted in incomplete films.

Figure 5.SEM of kafirinmicroparticle films after vigorous agitation in water for 72 h at 22^{o}C: **a**: Control; **b**: 10% GTA; **c**: 20% GTA; **d**: 30% GTA. Arrows point to the holes probably caused by physical damage by water agitation. GTA- Glutaraldehyde.

TABLES

Table 1. Effects of treating KafirinMicroparticles with Heat, Transglutaminase and Glutaraldehyde on the Protein Secondary Structure of Films prepared from them, as determined by FTIR

Treatment		Relative proportion of α-helical conformation at Amide I band (%)
Control	22ºC	53.9 e (0.1)
Heat		
	50ºC	52.2 d (0.9)
	75ºC	50.7 c (0.1)
	100ºC	48.0 a (0.6)
Transglutaminase		
	Maltodextrin	50.8 c (0.7)
	0.1% TG + Maltodextrin	50.4 c (0.4)
	0.3% TG + Maltodextrin	49.3 b (0.3)
	0.6% TG + Maltodextrin	48.5 a (0.5)
Glutaraldehyde		
	10%	50.6 c (0.5)
	20%	51.0 c (0.9)
	30%	52.2 d (0.3)

Values followed by different superscript letters are significantly different ($p<0.05$). Numbers in parentheses are standard deviations (n=4). Control for heat and glutaraldehyde treatments is the same. Amide I band (≈1650–1620 cm^{-1}). TG- Transglutaminase.

Table 2. Effects of Glutaraldehyde Treatment on the Water Uptake and Weight Loss in Water of KafirinMicroparticle Films

Glutaraldehyde treatment	Water Uptake of film (g/100 g dry film)	Film Weight Loss in water (g/100 g dry film)
Control	29.2 c (0.4)	31.8 b (0.6)
10%	27.0 b (1.2)	29.9 a (0.9)
20%	26.0 b (0.9)	29.3 a (0.4)
30%	23.4 a (1.4)	29.1 a (0.3)

Values in the same column but with different letters are significantly different ($p<0.05$). Numbers in parentheses are standard deviations (n=3).

Table 3. Effects of treating KafirinMicroparticles with Heat, Transglutaminase and Glutaraldehyde on the Surface Density, Thickness, Tensile Properties and *In Vitro* Protein Digestibility (IVPD) of Films made from them

Treatment		Surface Density (mg/cm^2)	Thickness (μm)	Maximum Stress (N/mm^2)	Maximum Strain (%)	IVPD (%)	IVPD (Percentage of control)
	Before soaking in water						
Control	22°C	3.42 a (0.01)	16.3 ab (1.0)	24.3 e (5.2)	2.6 a (0.2)	37.7 e (0.8)	
Heat							
	50°C		20.3 c (0.9)	18.6 cd (0.7)	3.5 ab (1.1)	37.1 e (0.3)	98.4
	75°C		19.6 bc (0.3)	13.5 ab (0.5)	2.6 ab (0.6)	36.6 de (0.2)	97.1
	100°C		21.0 c (0.4)	10.7 a (1.4)	3.0 ab (0.4)	35.0 cd (0.8)	92.8
Transglutaminase							
	Maltodextrin		22.7 cd (3.3)	19.7 d (1.1)	2.5 a (0.5)	34.0 bc (0.6)	90.2
	0.1% TG + Maltodextrin		25.3 de (0.3)	15.0 bc (0.5)	2.1 a (0.6)	32.8 b (1.2)	87.0
	0.3% TG + Maltodextrin		28.0 e (1.1)	10.2 a (1.2)	2.1 a (0.4)	31.0 a (1.0)	82.2
	0.6% TG + Maltodextrin		26.5 e (0.6)	9.3 a (0.2)	2.0 a (0.6)	30.3 a (0.4)	80.4
Glutaraldehyde							
	10%	3.45 ab (0.02)	16.3 ab (2.4)	34.8 f (3.7)	4.1 b (0.4)	39.7 f (0.9)	105.3
	20%	3.49 b (0.02)	15.8 a (1.7)	13.9 b (2.1)	12.2 c (0.4)	40.7 fg (1.2)	108.0
	30%	3.56 c (0.04)	16.1 ab (3.6)	14.2 b (2.6)	13.9 d (2.1)	42.2 h (0.2)	111.9
	After soaking films in water for 24 h and drying in air at ambient temperature for 30 min						
Glutaraldehyde							
	Control			6.6 a (2.2)	0.8 a (0.2)		
	10%			11.8 b (1.1)	1.6 b (0.2)		
	20%			16.2 b (4.4)	1.7 b (0.5)		
	30%			12.7 b (4.2)	1.7 b (0.4)		

Values in a column followed by different letters are significantly different ($p<0.05$). Numbers in parentheses are standard deviations (n=3). Control for heat and glutaraldehyde treatments is the same. TG- Transglutaminase. At least four film strips were analyzed per film giving at least 12 film strips per treatment.

FIGURES

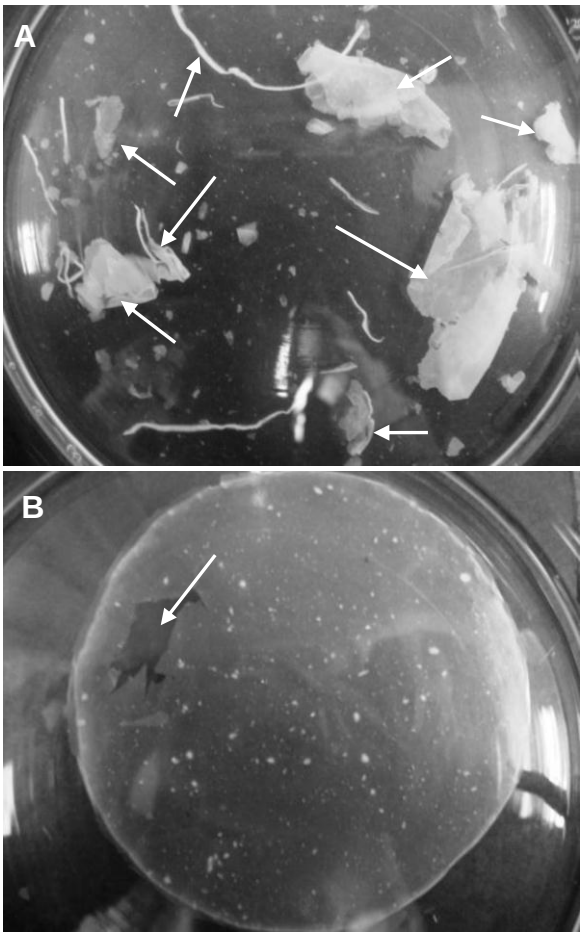
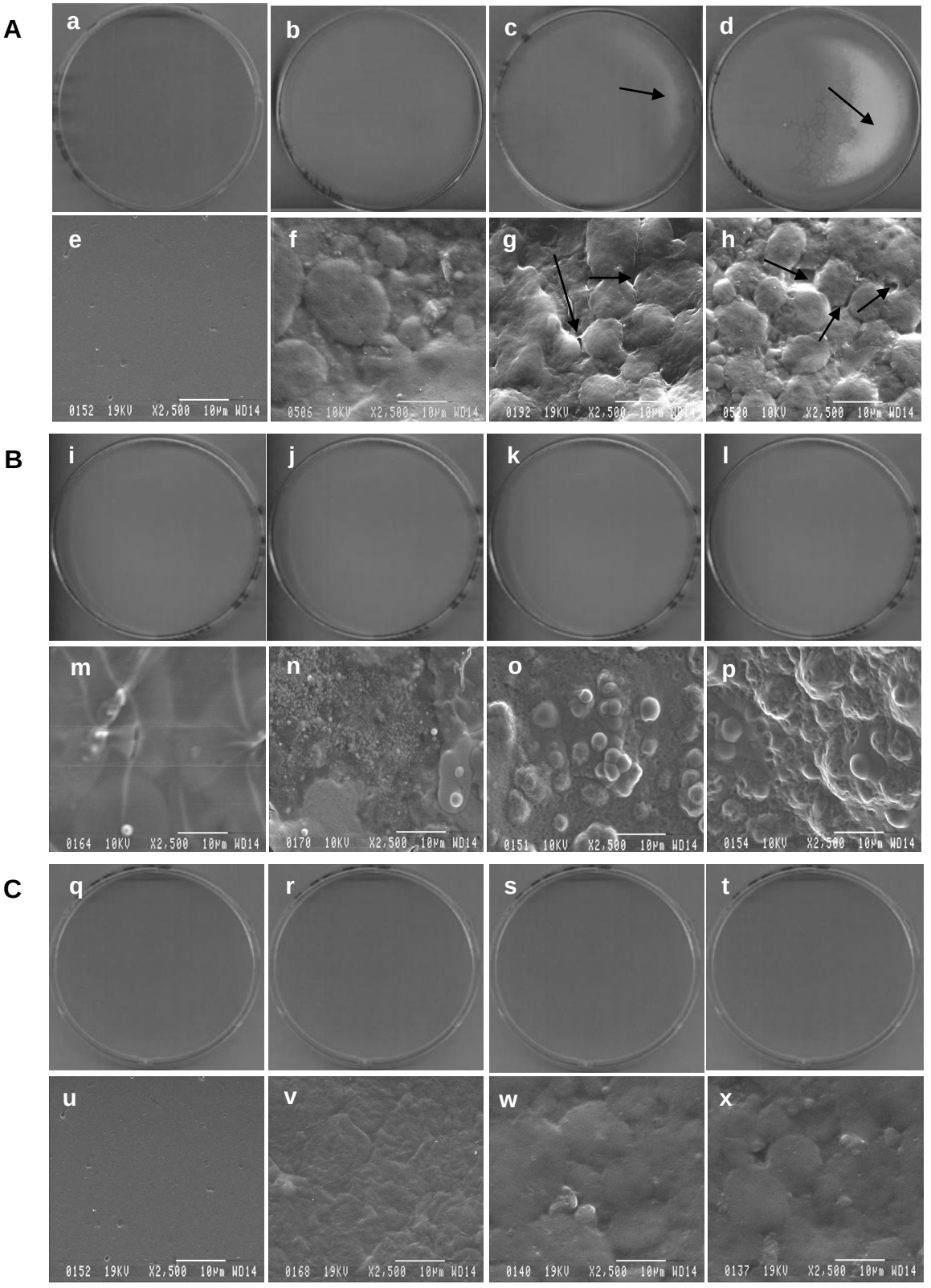


Figure 1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a
b
c
d
e
f
g
h
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0520 10KV X2,500 10μm WD14
B
i
j
k
l
m
n
o
p
0164 10KV X2,500 10μm WD14
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0151 10KV X2,500 10μm WD14
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C
q
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0152 19KV X2,500 10μm WD14
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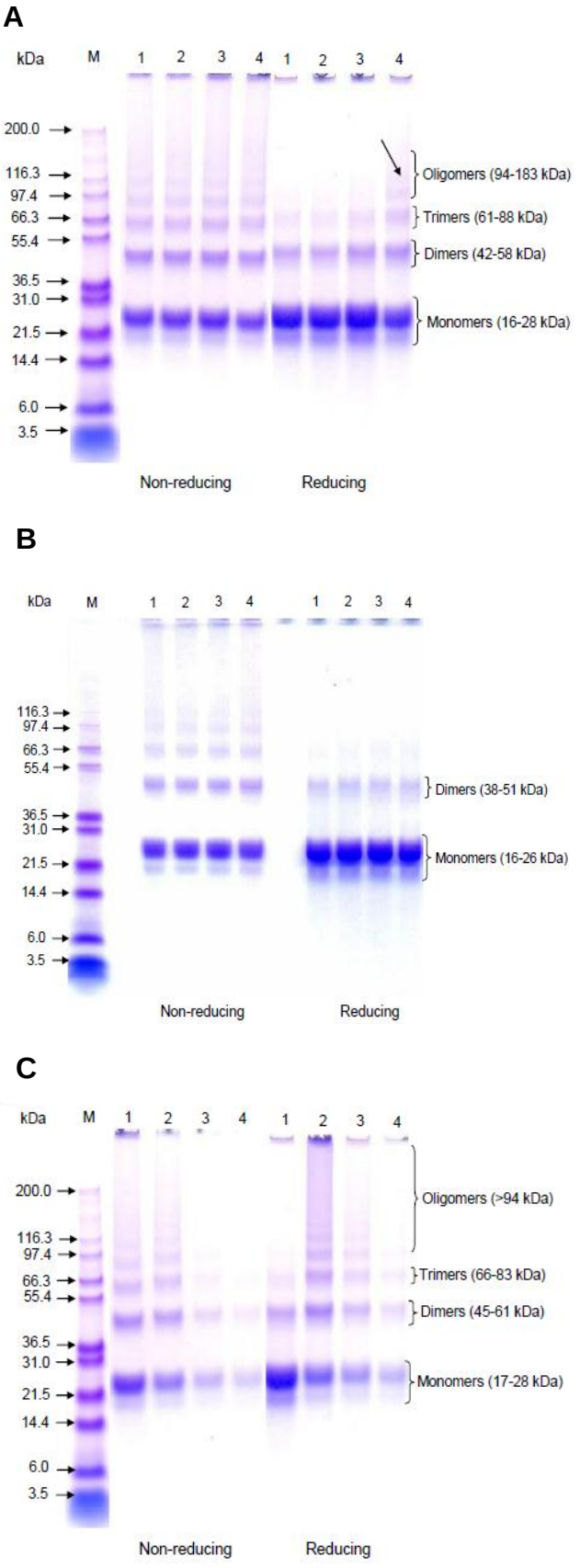
Figure 2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kDa
M
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200.0
116.3
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0
3.5
Oligomers (94-183 kDa)
Trimers (61-88 kDa)
Dimers (42-58 kDa)
Monomers (16-28 kDa)
Non-reducing
Reducing
B
kDa
M
1 2 3 4 1 2 3 4
116.3
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0
3.5
Dimers (38-51 kDa)
Monomers (16-26 kDa)
Non-reducing
Reducing
C
kDa
M
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200.0
116.3
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0
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Trimers (66-83 kDa)
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Monomers (17-28 kDa)
Non-reducing
Reducing

Figure 3

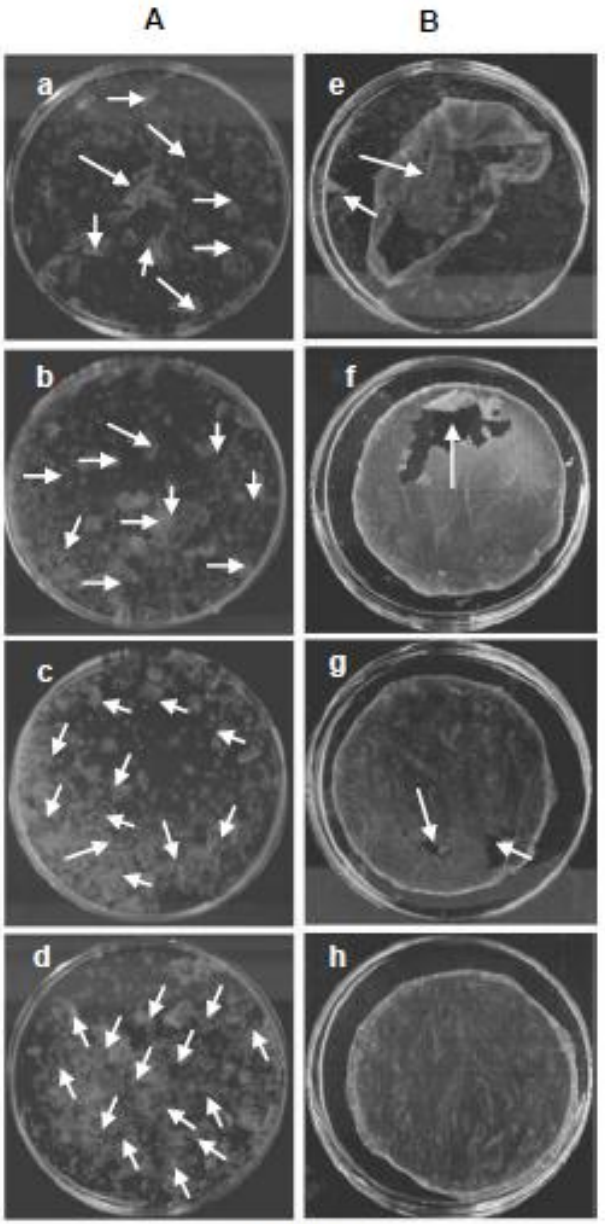

Figure 4

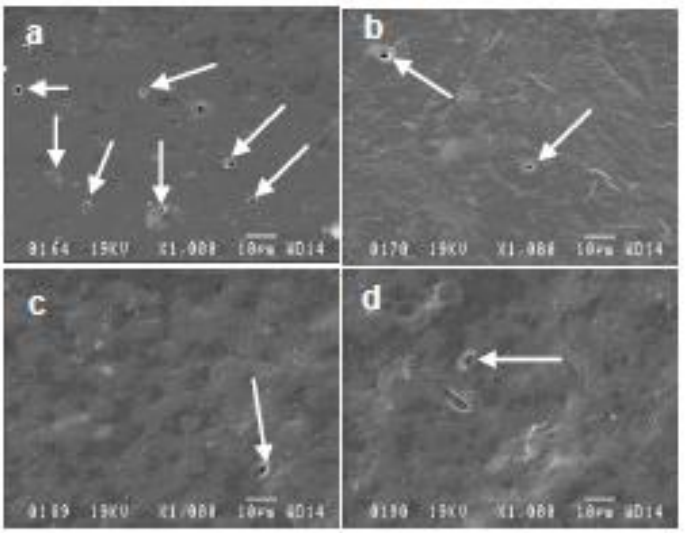

Figure 5