

5 GENERAL DISCUSSION

The general discussion is divided into three subsections. The first is a critical review of the main methodologies as applied in this research, including cross-linking techniques, electron microscopy, AFM, SDS-PAGE, FTIR, ELISA and subcutaneous rat bioassay. Mechanisms responsible for cross-linking of the kafirin microparticles and how they relate the morphologies, especially the occurrence of spindle-shaped glutaraldehyde-treated kafirin nanostructures will be proposed. Lastly, there will be a brief discussion on the potential application of the modified kafirin microstructures and suggestions for future studies that might be required to optimize the application of these microstructures. Reviews of the methodologies used in the measurement of water vapour barrier and tensile properties were discussed by Emmambux (2004) and so will not be reviewed further here.

5.1 METHODOLOGY: CRITICAL REVIEW

Cross-linking treatments were performed on pre-formed kafirin microparticles. As discussed, an attempt to modify the microparticle structures with heat or glutaraldehyde while forming them produced only slightly larger microparticles compared to the control. In addition, there was absence of vacuoles within the microparticles cross-linked with heat during their preparation, while there was a reduction in vacuole size with glutaraldehyde treatment. However, when the treatments were applied on pre-formed microparticles, there was a significant change in particle morphology, hence this protocol was applied. Cross-linking the kafirin in solution in glacial acetic acid before preparation of the microparticles was considered but applying the cross-linking treatments directly to kafirin dissolved in glacial acetic acid would have been impractical. First, heating kafirin in high acid concentration could probably decompose the protein through acid hydrolysis, as has been reported with other proteins (reviewed by Tsugita and Scheffler, 1982; Fountoulakis and Lahm, 1998). Secondly, the glutaraldehyde cross-linking reaction would probably be less effective at high acid concentration due to instability of Schiff base (imine) formed by interaction of the aldehyde carbonyl group and amino group from the protein (reviewed by Migneault et al., 2004). Additionally, as the optimal pH for the transglutaminase enzyme reaction is 6–7 (Ando et al., 1989) a low pH would have inactivated the enzyme. Another challenge was that the temperature used in the present study for transglutaminase reaction was 30°C, which is

well below the optimum of 50°C (Ando et al., 1989). This was necessary to preclude heat-induced disulphide cross-linking of the kafirin proteins as this would have confounded interpretation of data from the enzyme treatment, especially because 50°C was used as a temperature for heat treatment. But the application of the lower temperature may have compromised transglutaminase catalysed cross-linking reaction. However, the lower temperature did not affect the precision of the findings.

Conventional SEM was used to examine the surface properties of the microparticles and microparticle films. While conventional SEM protocol does not invalidate data, its main limitation is the potential introduction of artefacts due to sample preparation. As the microparticles were wet, they had to be dried before examination hence the images obtained were not necessarily of 'native' state kafirin microparticles. Therefore, the SEM images showing association (agglomeration) of particles may possibly have been a sample preparation artefact arising from removal of the acid. Drying would therefore probably result in sphere-to-sphere interfaces resulting into particle agglomeration. Therefore, no conclusive evidence could be drawn from such observations, especially with the transglutaminase-treated microparticles. In addition, there was poor image resolution at relatively high magnification ($\times 10000$). Thus, the magnification was limited to $\times 2500$ for clarity of the SEM images. Lastly, using SEM in an attempt to investigate the effect of BMP-2 binding on the morphology of the kafirin microparticles did not show physical difference, probably because of the very low proportion of BMP-2 compared to the binding material, kafirin microparticle or collagen standard.

TEM was used to study the internal microstructure of the microparticles. A drawback in the use of TEM is also the potential occurrence of artefacts, which may result from specimen preparation, such as staining, drying and sectioning-induced artefacts (reviewed by Talmon, 1983; Vinson, Talmon and Walters, 1989). In particular, thin sections of samples which are normally used to prevent excessive inelastic scattering of the electron beam (reviewed by Vinson et al., 1989), made the sections weak, resulting in tearing of resin that holds the samples in place, thereby compromising the quality of some of the TEM micrographs. Nonetheless, TEM provided very useful fine details of the internal structure of the microparticles, particularly by providing information on the effects of cross-linking treatments on the microparticle vacuole sizes.

AFM was used to study the structure of the kafirin microparticles at nanoscale and to measure their mechanical properties. One challenge that was encountered with AFM images was the broadening effect, in which some sections of the micrographs showed apparently larger nanostructures than the others. This meant that the acquired surface topography image could sometimes not accurately correspond to the real surface features. The broadening effect is a well-known artefact with AFM (reviewed by Shakesheff et al., 1994). It is due to the tip-sample convolution, which results when radius of curvature of the tip is similar to or greater than the size of the feature that is imaged. Image broadening is common in rougher areas of the sample where the broader end of the tip is involved in imaging (reviewed by Shakesheff et al., 1994; Shibata-Seki, Masai, Tagawa, Sorin and Kondo, 1996). In the present study, estimated nanostructure sizes were obtained from the flat areas, which have been shown to give a more accurate approximation of particle size (Shakesheff et al., 1994). AFM at higher resolution (<100 nm) was avoided as it resulted in poor image focus. Higher resolution would have required a smaller tip radius (reviewed by Weihs, Nawaz, Jarvis and Pethica, 1991). In this study, the smallest tip available was of 8 nm radius. Despite the broadening artefact with AFM, the existence of numerous published studies using this technique demonstrates that it still a reliable method for nano scale study of biomaterials.

Another challenge found with the use of AFM was in the determination of mechanical properties of the kafirin microparticles. The drawback in this case was that the AFM technique probed a very small section of the microparticle. Hence, it was incapable of direct mechanical characterization of a whole microparticle. This is a result of the fact that the AFM cantilever tip used was 9 nm which is very small compared to kafirin microparticles, which were >1 μm . Therefore, the mechanical properties of the protein microstructures determined by the AFM technique were limited to a localized probing of mechanical parameters, which were then used to provide general information on the mechanical strength of the whole particle. In addition, the AFM technique was limited to a narrow force measurement range (nanoscale range). The use of a force-feedback microelectromechanical systems (MEMS) microgripper has been suggested as a technique for directly measuring the mechanical parameters on a whole microparticle by applying a micro-scale compression testing (reviewed by Kim, Liu, Zhang, Cheng, Wu and Sun, 2008; Kim et al., 2010). However, this equipment was not available during the present study. It may be important to note that while the use of the force-feedback MEMS microgripper would have provided a direct measurement of the general mechanical strength of the microparticles, in principle the overall

mechanical properties of the particles are directly influenced by the local mechanical parameters of the components of the microparticles (nanostructures). In fact, the profiles of the force-deformation curves obtained in the present study using AFM were generally similar to those obtained with the force-feedback MEMS microgripper when used to measure the mechanical properties of biomaterials (Kim et al., 2008). In addition, despite these limitations, biomaterial mechanical characterization technique using AFM has been used in many studies, such as in the determination of mechanical strength of insulin microparticles (Volodkin, Schmidt, Fernandes, Larionova, Sukhorukov, Duschl, Möhwald and Von Klitzing, 2012). However, as discussed, the AFM data on the microparticle mechanical properties have to be interpreted with caution in light of the fact that the method measured only a very small portion of the microparticles.

FTIR was used to determine the secondary structure of kafirin polypeptides in the microparticles. The Amide I band was used to study the secondary structure. As stated, the Amide I band is more reliable than the other Amide bands as 80% of it is due to one functional group, which is the C=O, of the protein (Pelton and McLean, 2000). A limitation of the use of FTIR in this work was that despite significant changes in the physical appearance and functional properties of the kafirin microstructures as a result of transglutaminase and glutaraldehyde treatments, the alterations in protein secondary structures due to these two treatments were rather small. This limitation of FTIR probably arises from the fact it is relatively a low-resolution spectroscopic method. Hence, it cannot establish the precise three-dimensional location of individual structural elements (reviewed by Surewicz et al., 1993).

Another limitation was that the FTIR data were presented as relative proportions of the α -helical conformations at the Amide I band. The heights of peak assigned to different types of secondary structure (i.e., α -helices and β -sheets) were measured to represent percentages of these structures in a given protein. As explained by Surewicz et al. (1993) this procedure provides only an estimate of protein secondary structure but not the absolute content of the various types of conformations. An alternative method would have been to apply X-ray crystallography (reviewed by Smyth and Martin, 2000) to get information on protein three-dimensional structure at high level of resolution. However, a challenge was the feasibility of X-ray crystallography of kafirin polypeptides, as a crystallographic study would have required high-quality single kafirin protein crystals that were not available. This is probably

because the hydrophobic nature of these proteins would lead to amorphous aggregation in aqueous solutions, rather than crystal formation. Furthermore, the relatively “static” structure in single kafirin protein crystals would not have adequately represented the protein conformation in a complex and dynamic environment that existed in a microparticle or film.

SDS-PAGE, which separates protein molecules based on their sizes (reviewed by Yada et al., 1996), was used to determine the molecular weight distribution of protein in the kafirin microparticles and kafirin microparticle films. A limitation of this method was that despite evidence of reduction of protein digestibility with heat treatment, the SDS-PAGE did not expressly show clear difference in polymerization between the control and the heat-treated kafirin microstructures, except at the extreme temperature treatment. This is probably because kafirin polymerization may also occur as a result of oxidation (reviewed by Duodu et al., 2003). An alternative approach would have been to measure the amounts of free thiol (–SH) groups using 5,5′-dithiobis-(2-nitrobenzoate) (DTNB). Heat induced disulphide cross-linking reduces the amount of free –SH groups in kafirin (Elkhalifa, Bernhardt, Bonomi, Iametti, Pagani and Zardi, 2006; Ezeogu, Duodu, Emmambux and Taylor, 2008).

It is also a concern that the SDS-PAGE data with transglutaminase treatment did not correspond with the observed changes in the physical properties of kafirin microstructures. As discussed, apart from the transglutaminase cross-linking reaction that requires lysyl side chain, the other two reactions that are catalysed by transglutaminase are deamidation and amine incorporation (Motoki and Seguro, 1998). As all these three transglutaminase-catalysed reactions result in an increase in free ammonia, measurement of ammonia may be used as a tool to monitor the overall transglutaminase reaction (Sharma, Lorenzen and Qvist, 2001). However, it is important to point out two weaknesses of this particular method. The measurement of ammonia neither differentiates among the different transglutaminase reactions nor directly measures the cross-linking effect, which was of interest in the modification of the functional properties of kafirin microstructures. A method that directly measures the cross-linking reaction is the estimation of ϵ -(γ -glutamyl)-lysine, the isopeptide formed during the transglutaminase reaction, as applied by Sharma et al. (2001). As this isopeptide is not broken down by proteases that hydrolyse ordinary peptide bonds (Miller and Johnson, 1999), its estimation is possible by measuring the free amino acids once the other peptides have hydrolysed. However, in the present study as kafirin is very poor in lysine, getting meaningful transglutaminase-catalysed cross-linking data with this procedure would

have been unlikely. Hence, these proposed techniques should be complemented by another technique such as size exclusion chromatography (SEC) as performed by Emmambux and Taylor (2009) who applied this technique to determine the effects of cooking on the sorghum and maize prolamin structure. The solvent should consist of 1.0 M urea and 0.02M potassium phosphate buffer (pH 3) dissolved into a liquid mixture of ethanol, water, and lactic acid at a concentration of 5:2:3 (w/w), respectively. According to Emmambux and Taylor (2009), this solvent system results in negligible amount of undissolved kafirin.

A problem was also encountered with SDS-PAGE of kafirin microparticles and collagen bound to BMP-2. Initially Coomassie Brilliant Blue staining was used but no difference was found between the bands. This was thought to be due to the very low concentration of BMP-2 with respect to the binding protein, probably making it impractical to detect possible changes in the molecular weight distribution. Then silver staining, which works on the principle that proteins bind silver ions, which can be reduced under appropriate conditions to build up a visible image made of finely divided silver metal (reviewed by Chevallet et al., 2006) was performed on the samples. It was hoped that the effect of BMP-2 binding on molecular weights would be detected due to the high sensitivity of silver staining (>100 times the sensitivity of Coomassie Brilliant Blue staining) (reviewed by Chevallet et al., 2006; Schägger, 2006). However, the very high sensitivity of silver staining resulted in occurrence of ghost (artefact) bands (reviewed by Hurkman and Tanaka, 1986), making it difficult to see differences. Hence, it was not possible to obtain meaningful data.

An ELISA type assay was used to measure the concentration of BMP-2 in supernatants after binding reactions, to determine the amount of BMP-2 bound to kafirin microstructures or collagen. The particular ELISA was relatively expensive. Hence, it was reserved for confirmatory testing for presence of BMP-2 in the supernatant following a general protein concentration measurement with Lowry protein assay. ELISA uses quantitative sandwich enzyme immunoassay technique in which a monoclonal antibody specific for the protein had been pre-coated onto a microplate (Engvall and Perlmann, 1972). A concern was the large standard deviations in the data obtained, which called for cautious interpretation of the results. This is an inherent limitation of ELISA. The main source of variability in ELISA is the variation in efficiency and uniformity of coating antigen binding or the antigen-antibody reactions (reviewed by Li, Gee, McChesney, Hammock and Seiber, 1989; Sebra, Masters, Cheung, Bowman and Anseth, 2006). Sebra et al. (2006) explained that the antigen detection

assays that rely on monolayer formation or physisorption methods to immobilize antibodies to surfaces have drawbacks due to variation in antibody coating stability and uniformity, and are often heavily dependent on substrate properties such as surface chemistry and roughness. An alternative more accurate technique for precise measurement of BMP-2 would have been mass spectrometry such as matrix-assisted laser desorption ionisation/time-of-flight mass spectrometry (MALDI/TOF-MS). This mass spectrometry technique has been applied in BMP-2 binding studies such as in the determination the binding of bioactive BMP-2 to nanocrystalline diamond by physisorption (Steinmüller-Nethl, Kloss, Najam-Ul-Haq, Rainer, Larsson, Linsmeier, Köhler, Fehrer, Lepperdinger, Liu, Memel, Bertel, Huck, Gassner and Bonn, 2006). However, it is much more expensive in comparison to ELISA. In addition, the instrumentation was not available and would have required considerable method development for kafirin as it cannot be crystallised and is not easily soluble. As the trends with the different treatments were consistent, the high standard deviations did not invalidate the ELISA findings.

Rat model assessment of safety, biodegradability and effectiveness of kafirin microparticle film-BMP-2 system in inducing bone formation was performed to establish whether the kafirin microstructures would have potential application as biomaterials for use as implantable medical devices to promote bone tissue regeneration both in animals and in people. A subcutaneous implantation site was chosen in order to rule out osteoconduction or periosteal bone formation as disturbing mechanisms in the BMP-2 induced bone formation (reviewed by Kempen et al., 2008). The choice of a rat model for this study instead of larger animals was based on several reasons. First was the ethical concern with the use of larger animals, especially for early stage investigations such as the present study. Secondly, as explained by Seeherman, Wozney and Li (2002), with small animals there is an increased number of responding cells in the bone and soft tissue elements and a more rapid rate of bone formation.

There were some limitations with the rat model study in this work. The number of animals was less than the ideal full Organisation for Economic Cooperation and Development (OECD) toxicity studies requirement of 10 animals (five animals each of both sexes) per treatment (OECD, 1981). However, the application of four treatments (implants) per animal was meant to reduce the sample size (number of animals) (reviewed by Bessa et al., 2010), while providing baseline information on tissue inflammatory response to the implants. This

use of more than one implant site with different treatments per animal may not have been ideal, but studies have been performed with a similar experimental design with different animal models such as rats (Bessa et al., 2010) and rabbits (H.-J. Wang et al., 2007). Another limitation was the difference in the physical structures of the implant materials. The collagen was in particulate form, which was very different from the structure of the kafirin microparticle film. Perhaps a better comparison would have been achieved by using binding matrices with similar physical structures.

Also, immune sensitisation of the immunocompetent rats could have had limitations as regeneration of new bone cells may have been countered by osteolysis (Prof Vinny Naidoo, personal communication), which is an immune response to bone (Roodman, 1996; Jacobs, Roebuck, Archibeck, Hallab and Glant, 2001). Immunocompromised animals such as nude mice would have been better in this case. However, since this study was also meant to investigate the safety of the implants, it was thought that the use of immunocompromised rodents would be more appropriate at a later stage of investigation.

The short period of the study (four weeks) was also a limitation. It would have been better to perform a longer study (about 12 weeks) similar to that used by Kempen et al. (2008) who investigated the *in vitro* and *in vivo* biological activities of BMP-2 released from different delivery vehicles for bone regeneration. The samples would be drawn and examined at well-distributed intervals throughout the period of implantation. This probably would have provided more information on the degradability of the implant materials and their long-term effects in the animal body.

Another concern was that the rhBMP-2 dosages used in the present study were probably too low, as discussed (section 4.3.4.4). The different levels of rhBMP-2/BMP-2 dosage, different animal models and assorted durations of assessments to induce bone morphogenesis in the literature made it difficult to make a clear-cut comparison between the kafirin microparticle film-BMP-2 system and similar systems in literature. Other researchers such as Hollinger et al. (1998) working in similar animal assays have also cited this existence of a variety of experimental conditions in similar animal studies as a challenge in their work. In addition, studies also show that high dose of BMP-2 may induce structural abnormality of bone and inflammation in rats (Zara, Siu, Zhang, Shen, Ngo, Lee, Li, Chiang, Chung, Kwak, Wu, Ting and Soo, 2011). This complicated the decision on the right dosage, as rhBMP-2 appears to be more potent than partially purified BMP for regenerating bone in rats, as discussed.

There was also concern regarding some sample preparation techniques for histological evaluations. For example, embedding tissue implant materials with paraffin wax may not have secured the implant materials optimally for histological examinations. Therefore, during sectioning with a microtome some of the implanted material appeared to have pulled away from the tissue material, causing artefacts (Prof Resia Pretorius, Department of Physiology, University of Pretoria, personal communication). However, this was a normal tissue embedding technique applied by Idexx Laboratories.

Another concern was that the sterilization process used on the implant materials could have inactivated the rhBMP-2. Gamma-irradiation was selected as a sterilization method because it is generally used to sterilize BMPs. However, the effects of the methods commonly used for BMP sterilization (γ -irradiation and ethylene oxide) on the osteoinductivity of BMPs is controversial (reviewed by Pekkarinen, 2005). In the present study, the choice of γ -irradiation as sterilization method was motivated by suggestions in literature that γ -irradiation may have only slight effects on the osteoinductivity of BMP (reviewed by Pekkarinen, 2005). Other factors that could have caused disparity in results between the present study and other studies were differences in placement site of the implants and age of the animals (Prof Vinny Naidoo, veterinarian, personal communication). Animals respond differently to growth factors depending on the site of implantation as has been shown with animal models such as rats injected with growth factors at different sites (Fujimoto, Tanizawa, Nishida, Yamamoto, Soshi, Endo and Takahashi, 1999). Similarly, studies on the effects of age on the response of rodent animal models to bone growth factors has shown a better response of younger animals than older animals as was found in a study with rabbits (Critchlow, Bland and Ashhurst, 1994).

The activity of ALP, a zinc metalloprotein enzyme that splits off a terminal phosphate group from an organic phosphate ester (reviewed by Saraç and Saygılı, 2007), was measured as a marker for early osteoblast differentiation. Bone ALP level was expected to be elevated in case of an increased osteoblastic activity, as had been shown by other workers (Farley and Baylink, 1986; Van Straalen, Sanders, Prummel and Sanders, 1991; Stucki, Schmid, Hämmerle and Lang, 2001), due to an induction by BMP-2 in the implants. ALP activity assay has been used in previous similar studies such as those on rats (Eriksson, Nygren and Ohlson, 2004; Kempen et al., 2008), rabbits (H.-J. Wang et al., 2007), humans (Rosalki, Foo, Burlina, Prellwitz, Stieber, Neumeier, Klein, Poppe and Bodenmüller, 1993; Stucki et al.,

2001) and cell lines (Kraus, Deschner, Jäger, Wenghoefer, Bayer, Jepsen, Allam, Novak, Meyer and Winter, 2012). However, a limitation of ALP activity test in measuring bone morphogenesis was that the assay is not specific for bone growth as ALP is also formed by other organs such as the liver and intestines (reviewed by Hoffmann et al., 1994), as discussed. Nonetheless, previous studies have shown that bone ALP activity could be considered a marker of bone formation *in vitro* (Farley and Baylink, 1986) and *in vivo* (Van Straalen et al., 1991; Withhold et al., 1996). Another challenge was that the collagen implant material was digested by tissue enzymes. In contrast, the kafirin microparticle film implants remained largely intact. Therefore, the collagen implant material used for ALP test appeared to have a larger proportion of animal tissue material obtained at the implant site compared to film implant material, which may have confounded the results of the assay. Perhaps an alternative ALP assay using a staining technique such as Vector Red Alkaline Phosphatase Substrate as applied by Eriksson et al. (2004) would have provided a more useful information on the ALP activity. Despite the limitation, the ALP activity data seemed to correspond to the histology study, which showed essentially no bone morphogenesis. Hence, these limitations did not really affect the precision of the results.

5.2 PROPOSED MECHANISM OF CROSS-LINKING THE KAFIRIN MICROPARTICLES WITH GLUTARALDEHYDE AND HEAT

It was found that both heat and glutaraldehyde treatments resulted in larger kafirin microparticles (Chapter 1). Images based on surface morphology examined by AFM showed that kafirin microparticles seem to comprise coalesced nanostructures of different shapes, depending on the type of treatment. The glutaraldehyde-treated kafirin microparticles were apparently made up of spindle-shaped nanostructures, while round and irregular shaped nanostructures were obtained with heat treatment. The increase in kafirin microparticle size and difference in shapes of the nanostructures in the microparticles with heat and glutaraldehyde treatments indicated there was further assisted-assembly of kafirin polypeptides as a result of these treatments. The fact that the kafirin microparticles are apparently made up of nanostructures coalesced together evokes an analogy between the formation of kafirin microparticles and casein micelles. Casein micelles are formed by aggregation of sub-micelles (subunits made up of about 15–20 casein molecules each) and the stability of the casein micelle structure is maintained by κ -casein brush (a stabilizing coating) on the surface of the casein micelles (reviewed by Horne, 2006). Using the analogy

it could be presumed that kafirin nanostructures are stabilized by an external surface layer, probably constituted by the γ -kafirin polypeptides based on the model proposed by Taylor (2008) for kafirin microparticle formation using coacervation with acetic acid solvent. The Taylor (2008) model for kafirin microparticles formation, which is based on the structure of the spherical kafirin protein bodies (Shull, Watterson and Kirleis, 1992; Oria, Hamaker, Axtell and Huang, 2000), suggests that the α -kafirins are located at the centre of the microparticle followed by β -kafirin and then γ -kafirin layer, which stabilizes the kafirin microparticle. However, Taylor (2008) did not have data on details showing that the kafirin microparticles could be made up of nanostructures as observed in the present study. Given the similarity in scale of the sizes of the casein micelles (100–200 nm) and the kafirin nanostructures (100–300 nm), an analogy is drawn between the casein micelles and the kafirin nanostructures. Therefore, γ -kafirin polypeptide-enriched rich sub-nanostructures (subunits comprising a few molecules of kafirin sub-classes aggregating together) would be located on the surface of the kafirin nanostructures, while those sub-nanostructures deficient in γ -kafirin polypeptides would be located in the interior as shown in the proposed schematic representation (Figure 5.1A). These γ -kafirin polypeptide-rich sub-nanostructures would be analogous to the κ -casein rich casein sub-micelles as described by Horne (2006). As with casein micelle, the structure of the kafirin nanostructure would depend on the stability of the γ -kafirin-enriched polypeptide layer. Assuming that the kafirin microparticles produced by self-assembly are made of sterically stabilized kafirin nanostructures with a γ -kafirin polypeptide-enriched layer, one can relate the changes in the structure of the kafirin microparticles in response to heat or glutaraldehyde treatments by considering the behaviour of the γ -kafirin polypeptide-enriched layer in response to these treatments. This is by analogy with casein micelles where destabilizing the κ -casein brush by addition of the enzyme chymosin that breaks down the κ -casein brush, pH adjustment and addition of calcium or ethanol or combinations, leads to the collapse of the brush and thus flocculation of the micelles (reviewed by De Kruif, 1999).

With glutaraldehyde-treatment the spindle shape and unidirectional orientation of the nanostructures that seem to constitute the kafirin microparticles suggests that the mechanism of further assisted-assembly due to glutaraldehyde treatment was different from that with heat treatment. Only the main type of cross-linking between glutaraldehyde and proteins that involves the formation of Schiff bases will be discussed. The status of the γ -kafirin polypeptide-enriched rich region of the sub-nanostructures before and after collapse of the

kafirin nanostructures would influence the type of further assisted-assembly, in a similar manner to that reported with casein micelles (De Kruif, 1999). During the glutaraldehyde treatment it appears that destabilization of the γ -kafirin polypeptide-enriched layer occurs, thereby resulting in the loss of the round shape of the nanostructures as shown in the proposed schematic representation (Figure 5.1). The destabilization due to glutaraldehyde treatment probably results through formation of a γ -kafirin polypeptide-glutaraldehyde complex by covalent bonding (Figure 5.1B). This covalent bonding probably offsets the balance between the attractive forces (such as disulphide and hydrogen bonding) and repulsive (such as steric hindrance) forces between the different kafirin subclasses within the nanostructures that maintained the spherical shape. After the collapse of the spherical shapes of kafirin nanostructures, a realignment of sub-nanostructures that make up the nanostructures towards a particular direction could occur through a glutaraldehyde-assisted re-assembly driven predominantly by linear interactions as illustrated (Figure 5.1C).

The literature shows that linear aggregation of protein nanostructures requires the presence of hydrophobic rich domains and cross-linking domains as has been reported in several studies on elastin-like fibres (Bressan, Pasquali-Ronchetti, Fornieri, Mattioli, Castellani and Volpin, 1986; Bellingham, Lillie, Gosline, Wright, Starcher, Bailey, Woodhouse and Keeley, 2003; Osborne, Farmer and Woodhouse, 2008). In the present study, it is proposed that the linear attraction is likely to be driven by the kafirin polypeptide-glutaraldehyde linkage. As suggested by Migneault et al. (2004), the glutaraldehyde-protein reaction that results in a cross-linked structure consists of a linear aldol-condensed oligomer of glutaraldehyde linked to Schiff base from the protein. Treating the kafirin nanostructures with glutaraldehyde would initially result in cross-linking reaction with γ -kafirin polypeptide-enriched region on the surface of the nanostructures. Once the nanostructure has collapsed, possibly other kafirin subclasses become exposed resulting in glutaraldehyde cross-linking of the other kafirin subclasses. The formation of linear kafirin nanostructures requires the exposure of domains of the polypeptide that contain side chains that can be available for cross-linking thus promote aggregation by end-to-end alignment. In the cross-linking that results in formation of Schiff bases between carbonyl and amino-groups, glutaraldehyde preferentially reacts with basic amino acid residues that have free amino group (Migneault et al., 2004). As kafirin is poor in lysine, an alternative amino acid residue would be arginine.

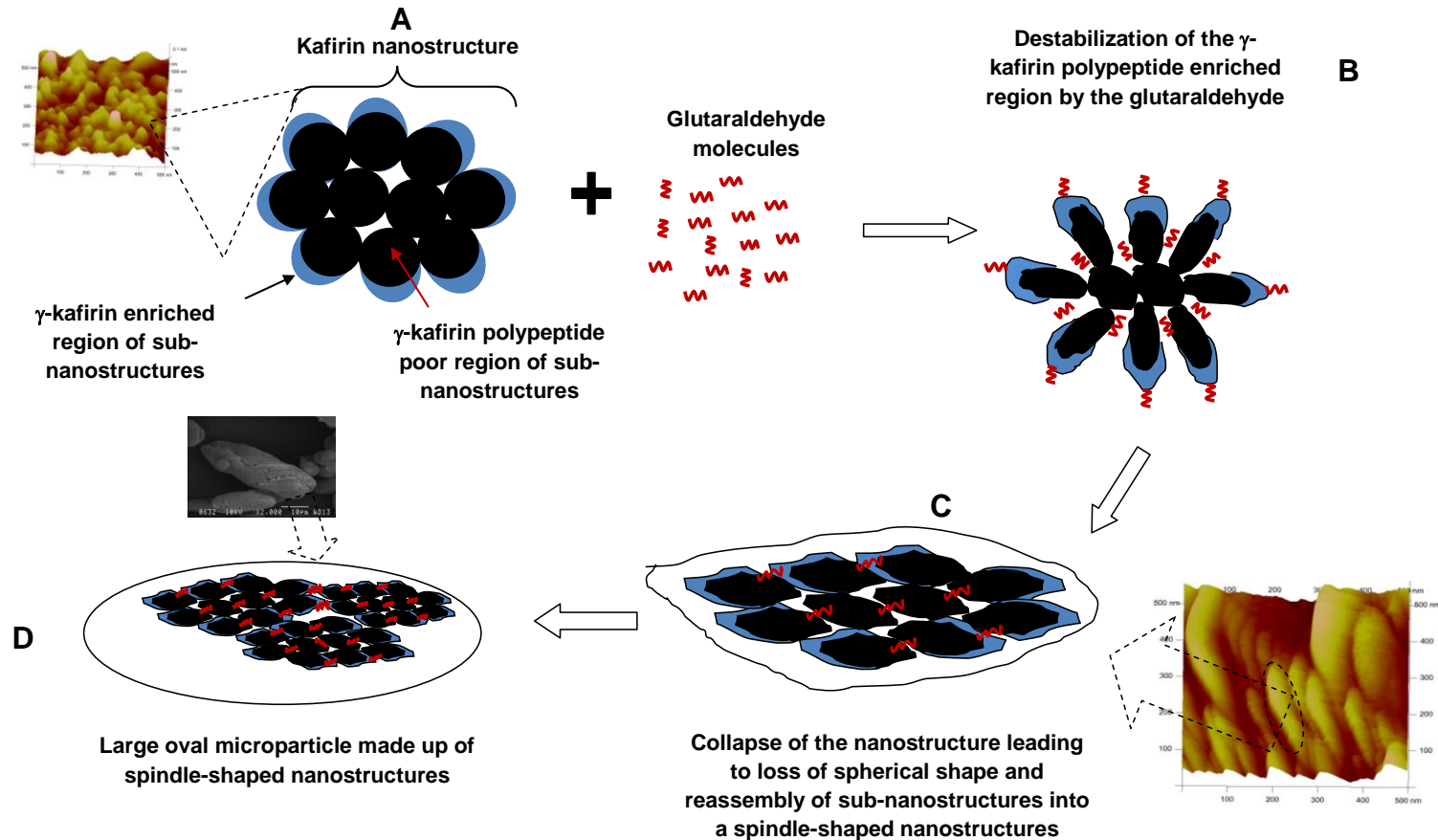


Figure 5.1 Schematic representation of proposed mechanism of glutaraldehyde cross-linking of kafirin microparticles (not drawn to scale). **A.** Kafirin nanostructure with the γ -kafirin polypeptide enriched regions of sub-nanostructures (subunits comprising a few molecules of kafirin sub-classes aggregating together) located on the surface, while regions deficient in γ -kafirin polypeptide are located in the interior. **B.** Destabilization of the γ -kafirin polypeptide-enriched layer. **C.** Loss of the round shape of the kafirin nanostructures and re-assembly of sub-nanostructures into linear spindle shapes driven by kafirin polypeptide-glutaraldehyde linkage. **D.** Cross-linking of adjacent kafirin nanostructures and merging into a large oval kafirin microparticle via kafirin polypeptide-glutaraldehyde linkage.

In addition, to achieve the hydrophobicity requirement for linear aggregation, treatment of γ -kafirin polypeptides with glutaraldehyde must reduce the number of hydrophilic groups when the aldehydes react with free amino groups. These would probably be at the basic amino acid domain Arg.His. of the γ -kafirin polypeptides, based on the amino acid sequences as given by Belton et al. (2006). Then, this in effect would increase the proportion and influence of the hydrophobic domains (with respect to the hydrophilic domains) in the γ -kafirin polypeptide, thereby favouring the linear assembly of the sub-nanostructures. The unidirectional aggregation of the kafirin nanostructures and elongation of kafirin microparticles as a result of cross-linking with glutaraldehyde also implies a sequential joining of about 500 to >2000 kafirin nanostructures. This is because measured lengths of more than 100 microparticles were distributed over a wide size range (5 to >20 μm) and the minimum length of glutaraldehyde treated nanostructure was about 100 nm. Additionally, to a less extent lateral aggregation (side-by-side association) of the kafirin nanostructures may arise from a nonspecific association, which is likely to occur between any hydrophobic sequences in the kafirin polypeptides. The increase in size of glutaraldehyde treated kafirin microparticles would result when adjacent microparticles are cross-linked through the kafirin polypeptide-glutaraldehyde covalent bonding and merging, as illustrated (Figure 5.1D).

With regard to heat cross-linking of the kafirin microparticles, the fact that the nanostructures were generally irregular in shape, apparently formed by merged spheres indicates that unlike with the glutaraldehyde treatment there was little (or less than in the case with glutaraldehyde treatment) destabilization of the γ -kafirin polypeptide-enriched layer, as illustrated in the schematic representation (Figure 5.2). This is probably because heat treatment could have reinforced the γ -kafirin coating through heat-induced disulphide cross-linking as depicted (Figure 5.2B). At nano level, the irregular shapes of the merged nanostructures through heat-treatment seems to suggest that there was a random disulphide cross-linking between γ -kafirin polypeptides on the surface of adjacent nanostructures within the same microparticle as result of the heat treatment (5.2C). The size of the microparticles increased, probably through heat-induced disulphide cross-linking of the γ -kafirin polypeptides on the surface of adjacent kafirin microparticles and merging into a larger kafirin microparticle (Figure 5.2D).

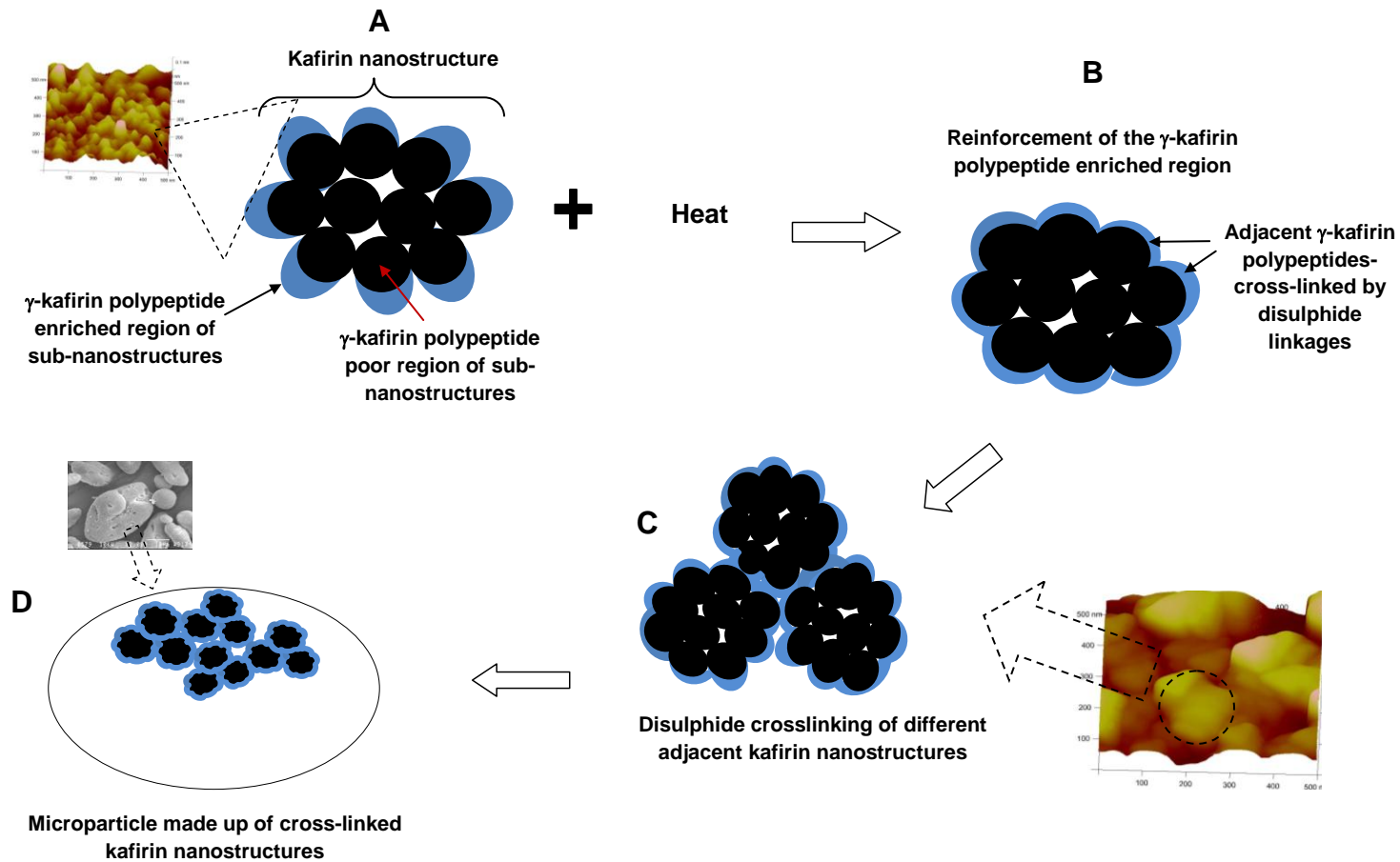


Figure 5.2 Schematic representation of proposed mechanism of heat cross-linking of kafirin microparticles (not drawn to scale). **A.** Kafirin nanostructure with the γ -kafirin polypeptide rich sub-nanostructures (subunits comprising a few molecules of kafirin sub-classes aggregating together) located on the surface, while areas deficient in γ -kafirin polypeptide are located in the interior. **B.** Reinforcement of the γ -kafirin coating through heat-induced disulphide cross-linking of adjacent sub-nanostructures. **C.** Heat-induced disulphide cross-linking of the γ -kafirin polypeptides on the surface of adjacent kafirin nanostructures. **D.** Merging of kafirin microparticles into a large microparticle.

The observed difference in shape of the smaller (spherical) and the larger (oval) heat-treated kafirin microparticles (section 4.1.4.1) was probably due to the rate of particle coalescence being inversely proportional to particle size, as proposed by Lehtinen and Zachariah (2001) who studied the effect of coalescence energy release on the temporal shape evolution of silicon and alumina nanoparticles. According to these authors, when two identical spherical nanoparticles coalesce as result of bonding between them a neck rapidly forms between the nanoparticles, which transforms into an oval shape that then slowly evolves into a sphere. As explained by these authors, the driving force for the transformation of two spherical nanoparticles into one completely fused particle is a minimization of the surface free energy reflected in a temperature increase of the resulting particle. As the characteristic coalescence time is inversely proportional to the solid-state diffusion coefficient, which is exponentially dependent on temperature, the heat release can significantly affect the overall coalescence process. This effect is significant especially for small particles since the fraction of their surface atoms is large and their overall heat capacity is small compared to larger particles. Therefore, the smaller particles are typically spherical, probably because the small size enhances coalescence rate, thereby reducing the time required to reach the final stages of shape evolution. In contrast, the larger particles have a higher heat capacity resulting in slow heating rate and slow evolution from an oval shape to a sphere. Hence, in the present study one would expect to see mainly non-spherical shapes for larger kafirin microparticles, as observed.

Cross-linking during kafirin microparticle formation resulted in relatively smaller microparticles compared to kafirin microparticles cross-linked after their formation (section 4.1.4.1). To explain the differences in size, the evidence from AFM images that showed that the kafirin microparticles are formed by coalescence of kafirin nanostructures could be applied. In the cross-linking during kafirin microparticle formation the building blocks are kafirin proteins, which are smaller in size. Therefore, this system is dominated by attractive forces between the kafirin polypeptides, which would result in smaller microparticles. In contrast, during cross-linking of pre-formed kafirin microparticles, the building units are larger as they are kafirin nanostructures. Hence, this system will be dominated by repulsive forces due to kafirin nanostructure crowding, which would result in larger microparticle size. This theory is analogous to that of Farrer and Lips (1999) who studied the mechanism of casein micelle formation from sodium caseinate. Their model demarcates two separate

regimes of close packing: below and above close packing. In this Farrer and Lips (1999) model, below close packing is expected to be sensitive to attractive interactions between sub-micelles and above close packing to be dominated by repulsive forces of micellar crowding/interpenetration.

There were differences between the sizes of the vacuoles within the kafirin microparticles cross-linked during microparticle formation and the vacuoles within microparticle cross-linked after microparticle formation. The reduction in vacuolation of microparticles cross-linked by heat during their formation was probably because of expulsion of air dissolved in the water by heat while the microparticles were being formed. In contrast, kafirin microparticles heat-treated after their formation had larger vacuoles as the air entrapped within them during their formation probably expanded with heat treatment. As discussed, air footprints have been suggested as the cause of vacuolation of the kafirin microparticles formed by coacervation (Taylor et al., 2009a). With regard to glutaraldehyde treatment, the reduction in vacuolation of the kafirin microparticles cross-linked by glutaraldehyde during their formation is probably because smaller microparticles would be dominated by attractive forces as discussed, probably resulting in reduction of vacuole sizes. On the contrary, the kafirin microparticles cross-linked after their formation were larger compared to microparticles cross-linked during their formation. Therefore, these larger glutaraldehyde cross-linked kafirin microparticles would be dominated by repulsive forces, probably resulting into larger vacuoles compared to the smaller microparticles treated with glutaraldehyde during their formation.

The glutaraldehyde-treated kafirin microparticle films had better tensile properties and water stability than control, probably mainly because of increase in covalent bonding within the film matrix as result of the kafirin polypeptide-glutaraldehyde linkages. In addition, it has been reported that the properties of the κ -casein brush, before and after collapse influences the fusion of the casein micelles thereby determining the gel properties (reviewed by De Kruif, 1999). Therefore, drawing from the analogy between the kafirin nanostructures and casein micelles and using the same reasoning, if glutaraldehyde treatment of kafirin microparticles caused a collapse of the γ -kafirin polypeptide-enriched layer as proposed, then the aggregation of the nanostructures would result in a cohesive film matrix. This would probably result in the better film tensile properties and water stability.

Another possible basis for the superiority in the quality of glutaraldehyde-treated kafirin microparticle film compared to heat-treated microparticle film may be drawn from knowledge of the possible mechanism of cast kafirin microparticle film formation with acetic acid solvent. Taylor et al. (2009c) suggested that kafirin microparticle film formation involves controlled aggregation of kafirin microparticles, followed by dissolution of the microparticles in acetic acid and drying into a cohesive film. However, the findings in the present study indicate that the aggregation is likely to be at the nanostructure level. By inference, the casting technique used to prepare the kafirin microparticle films is in principle an EISA of kafirin nanostructures. As discussed (section 4.3.4.1), EISA involves binary or tertiary solvents where preferential evaporation of one of the solvents changes the polarity of the solution, which drives the self-assembly of solutes (reviewed by Wang and Padua, 2012). As with the EISA work on zein microstructures with aqueous ethanol (70% ethanol in water) solvent (Wang et al., 2008; Wang and Padua, 2010), in the present study it is proposed that the increase in kafirin nanostructure concentration through evaporation of acetic acid solvent (25% acetic acid in water) would result in the nanostructures approaching each other. This would create sphere-to-sphere interfaces or spindle-to-spindle interfaces depending on the shape of the kafirin nanostructures. As the acetic acid solvent evaporates, the kafirin nanostructures would fuse together into a continuous film, which is in accordance with the Shin, Grason and Santangelo (2009) model for transformation of soft spherical materials. According to their model, as the concentration increases, the spheres approach each other and fuse into a bicontinuous network (sponge phase) and then into a lamellar phase. In the present study it is proposed that with the spherical heat-treated kafirin nanostructures, there would be larger interstitial spaces as illustrated (Figure 5.3A), while the packing of the spindle-shaped nanostructures would be more compact (Figure 5.3B). In addition, as the heat-treatment of kafirin nanostructures seems to enhance the stability of the γ -kafirin polypeptide-enriched layer, fusion of the kafirin nanostructures during film formation may be less cohesive. Furthermore, the bigger heat-cross-linked kafirin nanostructures may have been difficult to dissolve, thereby resulting in films with poor quality.

Generally, kafirin microparticles had higher IVPD than the films prepared from them. This may be attributed to the fact that the film formation protocol involved evaporation of the acetic acid solvent at a slightly elevated temperature (50°C) for a long time (12 h). The high temperature and long heating time may have resulted into further disulphide cross-linking of

the kafirin proteins, thereby reducing the IVPD of the films compared to the IVPD of the microparticles from which they were prepared.

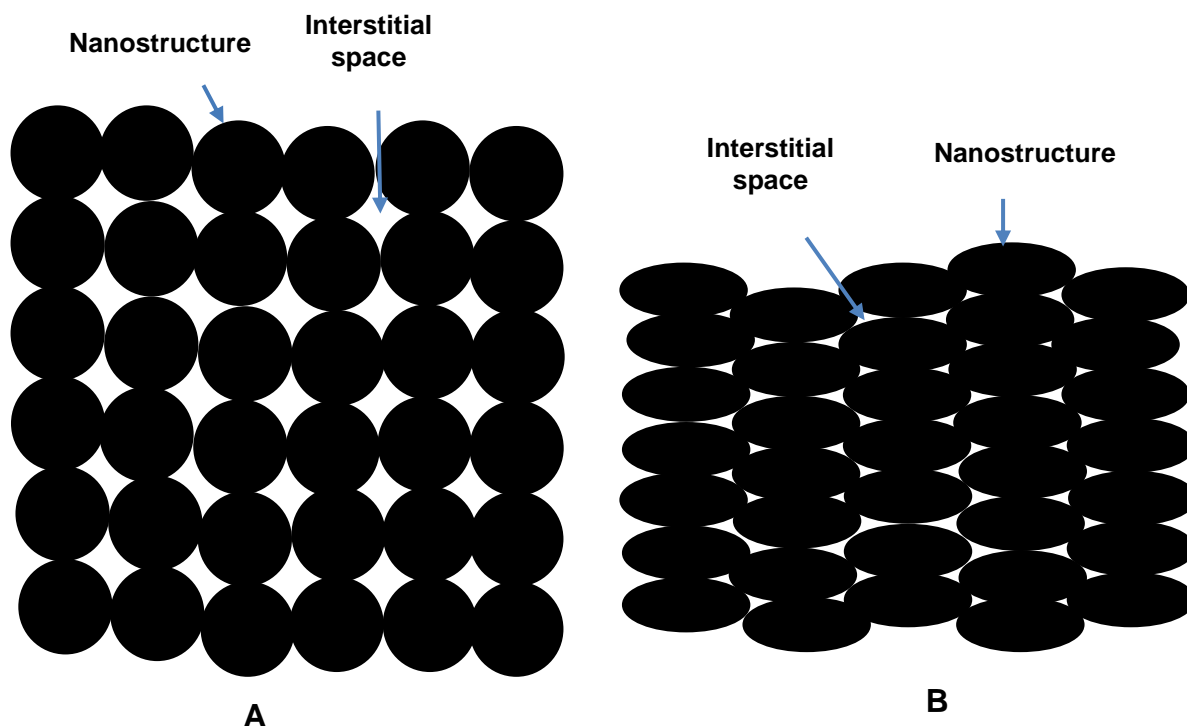


Figure 5.3 Schematic representation of hypothetical packing of kafirin nanostructures with different morphologies during film formation. **A.** Heat treated spherical nanostructures with large interstitial spaces. **B.** Glutaraldehyde-treated spindle- (oval) shaped nanostructures with smaller interstitial spaces forming a compact packing.

5.3 POTENTIAL APPLICATIONS OF KAFIRIN MICROSTRUCTURE BIOMATERIALS

The data on the safety, which indicated that the kafirin microparticle films were non-irritant when implanted in a rat model, gives kafirin microstructure biomaterials platform for many potential applications. For example, the increase in the size of modified kafirin microparticles obtained with heat and glutaraldehyde treatments makes them potentially suitable for application in scaffold-type structures for hard tissue repair that require large particles with a high degree of interconnected porosity. These kafirin microparticles could be implanted to fill a void in damaged bone tissue and subsequently seeded by the infiltration of the patient's own cells. As cells proliferate, deposition of extracellular matrix components and biodegradation of the kafirin microparticle scaffold would result in a solid, three-dimensional

tissue construct. Cell proliferation could be enhanced by loading the kafirin microparticle scaffolds with growth factors. In addition, the kafirin microparticle average size $\approx 20 \mu\text{m}$ obtained with heat and glutaraldehyde treatments makes them suitable as delivery devices for subcutaneous, intramuscular or intravitreal therapeutic drug administration. This is because the kafirin microparticle size is within the 10–250 μm size requirement that avoids microparticle uptake by macrophage phagocytosis and minimises inflammatory reaction (reviewed by Tran, Benoît and Venier-Julienne, 2011).

Kafirin microparticle films could be applied in skin tissue engineering for wound dressing to provide a bacterial barrier, control pain and contribute an adequate environment for epithelial regeneration due to their slow biodegradability and water stability. The kafirin microparticle films could be loaded with antimicrobial agents to avoid infection of the wound thereby enhancing the healing process. To treat an infected wound it is important to sustain sufficient drug concentration at the site of infection. Hence, the fact that kafirin microparticle film implants do not degrade rapidly could be utilised in their application as antimicrobial-loaded wound dressing.

The kafirin microparticle films could also be used in cell culture techniques. Cell culture techniques are important for the study of animal cell structure, function and differentiation and for the production of many important biological materials, such as vaccines, enzymes, hormones and antibodies. Cells could be grown on the surface of the kafirin microparticle films. The cells cultured on kafirin microparticle films could then be used as substrates for the production of viruses or cell products.

6 CONCLUSIONS AND RECOMMENDATIONS

Modification of kafirin microparticles with wet heat or glutaraldehyde treatment results in larger oval microparticles. In addition, heat treatment increases vacuole size. In contrast, transglutaminase treatment has little effect on the size of microparticles. While similar gross morphology of kafirin microparticles is obtained with heat and glutaraldehyde treatments, the shapes of presumably nanostructures that seem to coalesce to form the microparticles are very different. Heat treatment results in round-shaped nanostructures, indicative of non-linear protein aggregation in the kafirin nanostructures, probably due to disulphide cross-linking. In contrast, glutaraldehyde treatment results in spindle-shaped nanostructures, probably due to linear alignment of the kafirin nanostructures controlled by glutaraldehyde-polypeptide linkage. It is therefore apparent that with both treatments the pre-formed kafirin microparticle structures undergo some form of further assisted-assembly through different mechanisms. It seems that heat-induced disulphide cross-linking reinforces the layer, probably rich in γ -kafirin polypeptides, that stabilizes the spherical structure of the nanostructures within the kafirin microparticle. In contrast, glutaraldehyde-treatment appears to destabilize this structure-stabilizing layer through formation of γ -kafirin polypeptide-glutaraldehyde covalent bonding. This probably offsets the balance of attractive and repulsive forces between the different kafirin subclasses within the nanostructures, thereby resulting in collapse of the nanostructures and their linear realignment. Kafirin microstructures can bind BMP-2. The capacity of kafirin microparticles to bind BMP-2 is enhanced somewhat through modification by heat, transglutaminase or glutaraldehyde treatment, mainly due to increase in particle size. Thin glutaraldehyde-treated kafirin microparticle films are stable in water at ambient temperature, probably due to increase in covalent bonding within the film matrix through formation of the covalent glutaraldehyde-polypeptide linkage. Kafirin microparticle films are not toxic as kafirin is non-allergenic. The kafirin microparticle films also show a prolonged biodegradation when implanted in a mammalian tissue, probably because of the low susceptibility of kafirin to mammalian proteolytic enzymes. The large kafirin microparticles obtained with heat and glutaraldehyde treatments could have application as natural non-animal protein bioactive biomaterials for scaffold type structures used in hard tissue (e.g. bone) repair. Kafirin microparticle films could be applied in tissue engineering for soft tissue (e.g. skin) repair and in cell culture techniques for growing cells.

This study has provided valuable information on the mechanism for kafirin microparticle cross-linking with glutaraldehyde and heat. However, further work is needed in order to better understand the kafirin polypeptide self-assembly process at the molecular level so that the self-assembly process can be further manipulated to enable the formation of different structures. This will help in addressing the major challenges that remain such as production of microparticles with much larger vacuoles to allow cell migration throughout the structure, adhesion and proliferation to enable formation of functional tissues and organs. A related challenge is the production of larger films for use in large wound dressings. Research on the binding of kafirin microstructures with other types of bioactives such as nutraceuticals and antibiotic drugs is needed. A longer animal study with immunocompromised animal models to exclude the counter effects of immune sensitization in bone regeneration is recommended to provide information on long-term effect of the kafirin microstructure implants in animals.