1 INTRODUCTION

'Microparticles' is a collective name for microcapsules and microspheres, which are colloidal microstructures with size 1–250 µm (reviewed by Allemann, Leroux and Gurny, 1998; Reis, Neufeld, Ribeiro and Veiga, 2006). These microstructures can be made from synthetic polymers, natural biodegradable polymers, or a combination (reviewed by Sinha and Trehan, 2003). The common synthetic polymers used for preparing microparticles are polyesters such as poly(lactic acid) (PLA) or poly-lactic-*co*-glycolic acid (PLGA) (Freitas, Merkle and Gander, 2005), while examples of the natural polymers are proteins such as zein (Wang and Padua, 2010), kafirin (Taylor, Taylor, Belton and Minnaar, 2009a), albumin (Coombes, Breeze, Lin, Gray, Parker and Parker, 2001; MacAdam, Shaft, James, Marriott and Martin, 1997), gelatin (Mohanty and Bohidar, 2005) and polysaccharides such as chitosan (Guerrero, Teijón, Muñiz, Teijón and Dolores Blanco, 2010), alginate (X. Wang, Wenk, Hu, Castro, Meinel, Wang, Li, Merkle and Kaplan, 2007), starch (Heritage, Loomes, Jianxiong, Brook, Underdown and McDermott, 1996), cellulose (Kumar, Kang and Hohl, 2001) and dextran (Franssen and Hennink, 1998).

Most applications of microparticles are in the pharmaceutical industry, especially as carriers for drug delivery (reviewed by Tan, Choong and Dass, 2010). Microparticles can also be used in tissue engineering as scaffolds for cell transplantation to facilitate tissue regeneration for bone repair and for soft tissues regeneration in wound healing (Babensee, Anderson, McIntire and Mikos, 1998; Quirk, France, Shakesheff and Howdle, 2004). In the food sector, protein microparticles are used for improving flavour, aroma, stability, appearance, nutritional value and texture of food products (Stark and Gross, 1991). Microencapsulation has been used to incorporate health-promoting ingredients such as vitamins, antioxidants and essential minerals as well as fatty acids, into food (Schrooyen, Van der Meer and De Kruif, 2001). Protein microparticles have also been used to increase the survival of probiotic bacteria during their transit through the gastro intestinal (GI) tract by protecting bacteria in both the carrier food and in the human stomach (Marteau, Minekus, Havenaar and Huis In't Veld, 1997; Picot and Lacroix, 2004). It is projected that microparticles might be applied to improve absorption and bioavailability of nutrients and as nano-sensors for traceability and monitoring the condition of food during transport and storage (reviewed by Chaudhry, Scotter, Blackburn, Ross, Boxall, Castle, Aitken and Watkins, 2008).

Protein microparticles are natural products, which are biocompatible and normally have generally recognized as safe (GRAS) status (reviewed by Sinha and Tehran, 2003). Plant proteins in particular are widely available as by-products of food processing, such as the distillers dried grains with solubles (DDGS) which is the main co-product from grain-based ethanol production (reviewed by Wang, Tilley, Bean, Sun and Wang, 2009), brewers spent grains (Mussatto, Dragone and Roberto, 2006), brewing and milling (Lawton, 2002). Using proteins to make microparticles is technically advantageous as proteins are amenable to various chemical modifications that enhance microparticle surface hydrophilicity or hydrophobicity (reviewed by Chen, Remondetto and Subirade, 2006; MacAdam et al., 1997). For example, MacAdam et al. (1997) modified albumin microspheres to prepare hydrophobic and hydrophilic albumin microspheres with chemically reactive groups (carboxylic acid and amino residues) on the surface to which ligands could be attached and hence improve their drug delivery efficiency. In addition, proteins have natural capacity to bind potentially useful bioactive molecules (Wang, Yin and Padua, 2008). Such modifications may be necessary to improve the end-use functionality of protein microparticles. Microparticles produced from kafirin, the storage prolamin protein of sorghum, using simple coacervation with acetic acid as the solvent are vacuolated (Taylor et al., 2009a). This gives them a very large internal surface area, hence the ability to bind bioactive compounds as demonstrated with phytochemicals (Taylor et al., 2009b). These kafirin microparticles can also be used to prepare high quality free-standing bioplastic films (Taylor et al., 2009c). However, the quality of kafirin microparticles needs to be enhanced to broaden their potential applications and possibly to open up market opportunities for them.

2 LITERATURE REVIEW

In this review the term microparticles is defined in detail, and the principle behind the formation of protein microparticles is explained. Technologies for the preparation of protein microparticles are discussed. Properties of protein microparticles that are vital for their potential applications as well as the suitability of proteins to use for preparation of microparticles are discussed. A comparison between the functional quality of protein microparticles and other biodegradable microparticles is provided. Since kafirin will be the basic material in this research, a brief review of characteristics of kafirin proteins is given. Research into various methods used to improve the functionality of protein microparticles is reviewed. The principle behind the formation of protein microparticle films, techniques for their preparation and their quality compared to conventional protein films are described. A review is given of current and potential applications of protein microparticles and potential bioactives that can be bound or encapsulated by protein microparticles. Lastly, some of the techniques for studying the structures of protein microparticles are briefly discussed.

2.1 PROTEIN MICROPARTICLES

2.1.1 Definitions

Microparticles that have a shell and an inner core, in which active substances dissolve, although adsorption of these substances may also occur at their surfaces, are called microcapsules (Figure 2.1a). In contrast, microspheres have a matrix-type structure where active compounds can be adsorbed at their surface, entrapped or dissolved in the matrix (Figure 2.1b). Similar to microparticles, nanoparticles refer to nanocapsules and nanospheres with similar differences for capsules and spheres in the nanometre range. In this review, the term microparticles will be used for both unless specified otherwise. Microencapsulation refers to the process of enveloping a substance within another in a microscale (Schrooyen et al., 2001). In this thesis the term ‘microstructures’ will be used as a general term for protein structures including microparticles and films.

2.1.2 Formation of protein microparticles

There are two main ways to form protein microparticles. One approach involves reducing coarse protein powders to fine powders with microscale particle size through milling process

using ball mills, colloid mills, hammer mills and jet or fluid-energy mills (reviewed by Johnson, 1997).

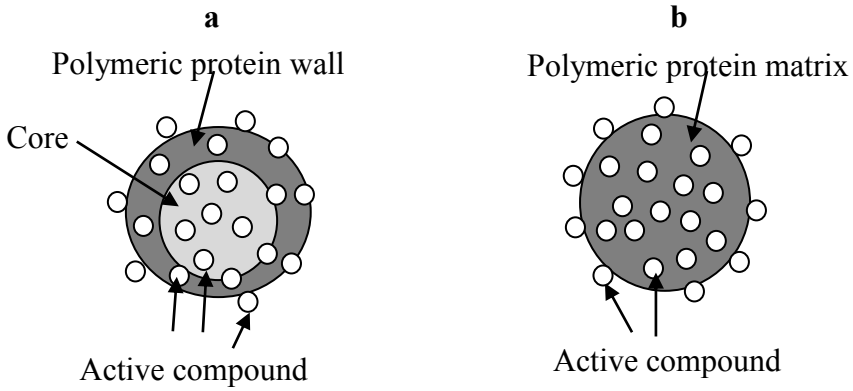

Figure 2.1 Schematic representation of protein microparticles. **a**. Microcapsules. **b**. Microspheres. Adapted from Reis et al. (2006).

Among these milling techniques, the jet mill is the most commonly used to prepare protein microparticles (reviewed by Shoyele and Cawthorne, 2006). Jet milling process involves micronization by interparticle collision and attrition, which typically produces particles size 1–20 µm (reviewed by Johnson, 1997). Alternatively, protein microparticles can be formed by molecular self-assembly (reviewed by Zhang, 2002; Lowik and van Hest, 2004; Wang and Padua 2010, 2012). Molecular self-assembly is the spontaneous organization of molecules under thermodynamic equilibrium conditions into structurally well-defined and rather stable arrangements (protein aggregates) (Whitesides, Mathias and Seto, 1991). As explained by Lowik and van Hest (2004), amphiphilicity is one of the main driving forces for self-assembly. Molecules containing both polar and apolar elements tend to minimize unfavourable interactions with the aqueous environment through a protein aggregation process, in which the hydrophilic domains become exposed and the hydrophobic moieties remain shielded. Self-assembly relies on weak non-covalent interactions, which typically include hydrogen bonds, ionic bonds and van der Waals' bonds. Protein aggregation usually irreversibly leads to the formation of amorphous, flocculent aggregates often heterogeneous in structure (Kentsis and Borden, 2004). As explained by Van der Linden and Venema (2007), the macroscopic and microscopic properties of materials that contain protein assemblies (aggregates) depend on the assembly structure, size, and interactions of the assemblies with the material matrix. Therefore, understanding the aggregation process of proteins may be useful in fabrication of protein microparticles for various applications. For

example, Wang and Padua (2010) working on zein used evaporation induced self-assembly (EISA) to form microspheres, packed spheres and films depending on zein concentration in aqueous ethanol solution. Wang, Crofts and Padua (2003) reported the formation of zein nanoscale tubes (cylinders) by self-assembly.

Protein aggregation is associated mostly with the presence of β-sheet conformation, as was found with zein aggregates (Mizutani, Matsumura, Imamura, Nakanishi and Mori, 2003). Militello, Vetri and Leone (2003) working on bovine serum albumin observed an increase in β-sheet structure at the expense of α-helical conformation after thermal aggregation of the protein. Yeo, Lee, Lee and Kim (2003) using silk protein, fibroin, compared the structures of pure amorphous fibroin and fibroin microparticles by studying their Fourier transform-infrared (FTIR) spectra. The amorphous fibroin sample had typical amide I and amide II bands at 1653 and 1531 cm^{-1}, respectively, which correspond to a random coil conformation. On the other hand, in fibroin microparticles, these peaks were shifted to lower frequencies, 1621 cm^{-1} and 1526 cm^{-1}, respectively, representing β-sheet conformation. The significance of β-sheet structure in the formation of protein microparticles has also been shown in kafirin microparticles (Taylor et al., 2009a). Despite the importance of the β-sheet conformation in aggregation, some proteins seem to deviate from this property as they maintain their native structure in their aggregated form. For example, human lithostathine has been shown to retain its native structure, during fibril formation through domain swapping (Laurine, Gregoire, Fandrich, Engemann, Marchal, Thion, Mohr, Monsarrat, Michel, Dobson, Wanker, Erard and Verdier, 2003).

Protein aggregation can be linear (also referred to as fibrillar) and non-linear (also known as particulate or aggregate) (reviewed by Van der Linden and Venema, 2007). The type of protein aggregation depends primarily on the general state of the protein when aggregation occurs (i.e. the concentration, temperature, pH, salt concentration, salt type and solvent added), rather than the specific amino acid sequence of the protein (Krebs, Devlin and Donald, 2007). These authors demonstrated this aspect when they worked with proteins that differ substantially from each other, but are all known to exhibit linear aggregation. They noted that after incubation at a pH near each protein's isoelectric point (pI), at an elevated temperature, all the proteins exhibited non-linear aggregation. Protein aggregations in foods are normally induced by heating and/or acidification. These treatments often induce unfolding of the proteins, thereby exposing certain sites, which are normally hidden within

the protein, and together with other properties triggers assembly (reviewed by Van der Linden and Venema, 2007).

Linear aggregation, also referred to as 'amyloid-like assembly', is created by linear association of protein molecules maintained by hydrophobic interactions (reviewed by Chen et al., 2006). It can occur through end-to-end aggregation of protein molecules (reviewed by Yeates and Padilla, 2002). Linear assemblies are formed when proteins are heated at pH values far from their pIs and at low ionic strength, when they are highly charged (Bolder, Hendrickx, Sagis and Van der Linden, 2006). The strong electrostatic repulsion that occurs under these conditions and the cross-β sheet structure imposes essentially a one-dimensional aggregation (Krebs et al., 2007).

Non-linear aggregation, also known as particulate or aggregate form is produced by random aggregation of structural units essentially controlled by van der Waals' forces (reviewed by Chen et al., 2006). The structural units are composed of large and almost spherical aggregates characterised by less elastic behaviour and lower rupture resistance. Particulate form occurs within pH range close to the proteins isoelectric point where net charge on the molecules is minimal (Krebs et al., 2007). This low charge, which offers minimal barrier to aggregation, together with partial unfolding of proteins at elevated temperature, results in fast and non-specific aggregation. Because the aggregation is non-specific, there is no directionality to the aggregates, resulting in the formation of three-dimensional spherical particles.

2.1.3 The technology for preparation of protein microparticles

The most common techniques for preparation of protein microparticles are spray-drying, solvent extraction/evaporation and phase separation/coacervation (reviewed Sinha and Trehan, 2003; Chen and Subirade, 2007). Other techniques are modifications of these three methods (reviewed by Freitas et al., 2005). The choice of the method depends on the properties of the protein and the purpose for which the microparticle is intended. Preparation method may be challenging when sensitive ingredients are to be encapsulated due to the possible damaging effect of formulation and process parameters, such as type of organic solvent, shear forces and temperature. Sinha and Trehan (2003) provided some factors to consider when selecting a method for encapsulation of active ingredients for controlled delivery. These include: optimum ingredient loading, high yield of microspheres, stability of the encapsulated material, batch uniformity and inter-batch reproducibility, adjustable release

profiles, low burst effect and free-flow properties of microspheres especially if they are intended for syringeable drugs. The most popular methods for the preparation of microparticles from hydrophobic polymers are organic phase separation and solvent removal techniques (reviewed by Herrmann and Bodmeier, 1998). A brief review of a few common techniques is dealt with next.

2.1.3.1 *Spray-drying*

In this process a feed protein solution is atomized into droplets that dry rapidly due to their high surface area and intimate contact with the drying gas (generally compressed air) (reviewed by Shoyele and Cawthorne, 2006). The solidified particles pass into a second chamber and are trapped in a collection flask. The advantages of this method include control of drug release properties of the resultant microspheres as well as the process being tolerant to small changes of polymer specifications (reviewed by Sinha and Trehan, 2003). Spray-drying is typically a process for preparing 1–10 µm size microspheres (reviewed by Sinha and Trehan, 2003) although slightly larger microparticles may be formed (Young, Sarda and Rosenberg, 1993). Young et al. (1993) used this technique to microencapsulate anhydrous milk fat using whey proteins. They observed that the whey protein capsules were spherical with smooth, wrinkle-free surfaces. Particle size ranged from 1–25 µm. However, this process requires high capital investment and has low recovery as it can result in huge losses through the exhaust vent (reviewed by Sinha and Trehan, 2003), which was noted using laboratory scale spray dryer (Yu, Rogers, Hu, Johnston and Williams III, 2002). Moreover, there is a risk of protein denaturation by physical stresses particularly high shear rates during atomization (reviewed by Johnson, 1997). Spray-drying technique is mostly applied in pharmaceutical industry for production of inhalant or aerosol drugs because it produces microparticles with suitable aerodynamic properties (Chan, Clark, Gonda, Mumenthaler and Hsu, 1997; Maa, Nguyen, Sweeney, Shire and Hsu, 1999).

2.1.3.2 *Solvent evaporation/removal*

In a typical solvent evaporation technique, the protein is dissolved in an aqueous organic solvent (Freitas et al., 2005; Tice and Gilley, 1985). An emulsion is formed by adding this suspension or solution to a vigorously stirring water (often containing a surface-active agent to stabilize the emulsion). The organic solvent is evaporated while continuing to stir. Evaporation results in precipitation of the protein, forming solid microcapsules containing

core material. When solvent removal method is used, the protein is typically dissolved in an oil miscible organic solvent (reviewed by Sinha and Trehan, 2003). The difference in this case is that the organic solvent is removed by diffusion into the oil phase while continuing to stir. No elevated temperatures or phase separating chemicals are required (reviewed by Sinha and Trehan, 2003; Freitas et al., 2005). Controlled particle sizes in the nano- to micro- metre range can be achieved. Cook and Shulman (1998) used this technique for preparation of zein microparticles.

2.1.3.3 *Phase separation/coacervation*

In this technique a protein solution is prepared and, while continuing to stir the solution, an *antisolvent* (a solvent in which the protein is insoluble) is slowly added to the solution (reviewed by Sinha and Trehan, 2003). Depending on the solubility of the protein in the solvent and antisolvent, either the protein precipitates or phase separates into a protein-rich and a protein-poor phase. Under suitable conditions, the protein in the protein-rich phase (coacervate) will migrate to the interface with the continuous phase. The proposed principle behind this technique is that when a protein solution is dispersed in an antisolvent, the binary mixture becomes a marginal solvent for the protein molecules, which precipitate (Mohanty and Bohidar, 2005; Taylor, 2008; Wang and Padua, 2010). Additionally, the poor solvation environment compels the protein molecules to reduce their spatial expansion thereby bringing charged protein functional groups ($-NH_3^+$ and $-COO^-$) to each other's vicinity through electrostatic interactions. This results in collapse of some single protein molecules through intramolecular interactions (self-charge neutralization), yielding the proteins microparticles with smaller radii. On the other hand, most other protein molecules associate (aggregate) to form large protein aggregates of larger radii through intermolecular electrostatic interactions. The morphology of the protein microparticles is attributed to the acquisition of a spherical shape needed for minimising the surface free energy through smallest surface area for a given volume if there is no contribution of other factors (Taylor, 2008).

Mathiowitz, Chickering III, Jong and Jacob (2000) working with zein developed an improvement of this technique in which the microparticle size was determined by non-stress parameters such as protein concentration, viscosity, solvent/antisolvent miscibility and solvent:antisolvent volumetric ratios. These workers claim that their modification is advantageous as it produces microparticles with minimal losses of the material to be encapsulated. This procedure produces microparticles of typical size 10 nm to 10 µm. The

microparticles produced by this technique are characterised by a homogenous size distribution and are thus expected to have well-defined, predictable properties. The phase separation technique has been used for preparation of zein microparticles for encapsulation of essential oils such as oregano, cassia and red thyme (Parris, Cooke and Hicks, 2005), for preparation of zein microspheres to deliver ivermectin (parasiticide) (Liu, Sun, Wang, Zhang and Wang, 2005), for preparation of ovalbumin-loaded zein microspheres (Hurtado-López and Murdan, 2005) and for preparation kafirin microparticles (Taylor et al., 2009a). The kafirin microparticles prepared by simple coacervation in which microparticles were expelled from a solution of kafirin in glacial acetic acid using distilled water were vacuolated (Taylor et al., 2009a). The existence of vacuoles greatly increases the surface area of materials, which may provide a matrix for the growth of cells (reviewed by Gong, Wang, Sun, Xue and Wang, 2006; H.-J. Wang, Gong, Lin, Fu, Xue, Huang and Wang, 2007) and binding with bioactive compounds such as polyphenols (Taylor et al., 2009b) and horseradish peroxidase (X. Wang et al., 2007). A disadvantage of coacervation is frequent impairment by residual solvents and coacervating agents found in the microspheres (reviewed by Freitas et al., 2005).

2.1.4 Properties of protein microparticles important for their application

To function appropriately, protein microparticles need to possess particular characteristics relevant to their intended use. A predominant use for microparticles is in encapsulation, which involves the envelopment of minute solid particles, liquid droplets or gases in a coating (Schrooyen et al., 2001; Reis et al., 2006). Encapsulation is aimed at providing delivery systems for various functional agents such as vitamins, minerals, antioxidants, polyunsaturated fatty acids and drugs. Weiss, Takhistov and McClements (2006) identified four functions that an effective delivery system must possess. First, it should carry the functional ingredient to the desired site of action. Second, in order to maintain the functional ingredient in its active state, it must protect the functional ingredient from physical, chemical or biological degradation during processing, storage, and utilization. Third, it should regulate the release of the functional ingredient, such as the release rate or the specific environmental conditions that trigger release (for example, pH, ionic strength, or temperature). Fourth, the delivery system has to be compatible with the other components in the system, as well as being compatible with the physicochemical and qualitative attributes (that is, appearance, texture, taste, and shelf life) of the final product. Protein microparticles intended for the food industry must be safe, non-toxic, edible and biodegradable. For biomedical applications, the

microparticles need to be biocompatible, resistant to possible attack by immune system *in vivo*, biodegradable and/or reabsorbable in the body once its function is accomplished and small enough for easy introduction into the body (Coombes, Lin, O'Hagen and Davis, 2003).

The size of protein microparticles appears to be a major factor that affects their applications. For example, when Liu et al. (2005) tested the suitability of zein microspheres for delivery of ivermectin, they found that ivermectin-loaded zein microspheres were suitable for use in drug targeting system because the diameter of the microspheres (0.3–1.2 μm) is appropriate for phagocytosis by macrophages. These workers stated, however, that microspheres with an average diameter of 1 μm were so small which may lead to a fast release because of the increased ratio of surface area to volume as the microspheres decrease in size. Chen and Subirade (2007) working on nutrient release properties of alginate–whey protein microspheres suggested that sustained release of encapsulated materials by larger particles is probably due to their smaller surface area/volume ratios and longer diffusion path lengths. On the other hand, syringeable drug-loaded microparticles are better if they are of smaller sizes.

The surface morphology of protein microparticles also affects their applications. Porosity of microparticles may be necessary for tissue engineering as it helps in tissue regeneration (reviewed by Karageorgiou and Kaplan, 2005; Gong et al., 2006). In addition, the presence of vacuoles (pores) within microparticles creates a large internal surface area for binding of bioactive compounds. For example, as discussed Taylor et al. (2009b) showed that vacuolated kafirin microparticles have the ability to bind antioxidants. The protein characteristics also influence the functionality of microparticles prepared from them. For example, studies using zein microparticles have shown that the zero-order release of ivermectin in the presence of pepsin (Liu et al., 2005) or sustained release of encapsulated lysozyme in an aqueous environment (Zhong, Jin, Davidson and Zivanovic, 2009), probably as a result of the the relatively high hydrophobicity of zein protein.

2.1.5 Quality of protein microparticles compared with other biodegradable microparticles

Synthetic biodegradable polymer microparticles are made from polyesters, polyanhydrides, polyorthoesters, polyphosphazenes, and pseudopolyamino acids of which polyesters have found most widespread application (reviewed by Sinha and Trehan, 2003). Synthetic microparticles have advantages over protein microparticles as they can be reproducibly

synthesized, easily purified and are well characterised (Hurtado-López and Murdan, 2005). However, the low toxicity, biodegradability and GRAS status of protein microparticles are their major advantage. In terms of performance, generally, synthetic polymer microparticles have higher active ingredients loading efficiency than microparticles prepared using natural polymers, probably enhanced by the longer period of their use and research. For example, Liggins, Toleikis and Guan (2008) produced polyethylene glycol (PEG) microparticles that could be loaded with a drug at a concentration of greater than 50% (weight drug/weight microparticle). High loading capacity may facilitate less frequent dosing and may increase efficacy. However, the use of synthetic polymers in the preparation of microparticles has some drawbacks. The instability of agent to be delivered (e.g. sensitive protein drugs) is one of the concerns (reviewed by X. Wang et al., 2007). Reviewing formulation aspects of polymeric biodegradable microspheres for antigen delivery, Tamber, Johansen, Merkle and Gander (2005) noted that the organic solvents used during microencapsulation and the acidic microenvironment generated during PLGA degradation can negatively affect the activity of many encapsulated proteins as well as labile molecules. In addition, there are some indications that the breakdown of PLGA and similar polymers to acids such as lactic and glycolic acids can lead to inflammatory reactions in some circumstances when used as scaffold materials (reviewed by Babensee et al., 1998; Quirk et al., 2004).

Concerning the use of natural non-protein polymers to prepare microparticles, some potential technical limitations may be encountered. For example, alginates, which are seaweed derived gel-forming polysaccharides comprising chains of alternating α-L-guluronic acid and β-D-mannuronic acid residues, can be used to prepare biocompatible drug carriers. This can be done through controlled gelation by chelation between the carboxyl groups of its α-L-guluronic acid residues with either Ca^{2+}, Ba^{2+} or poly(L-lysine) (Draget, Skjåk-Bræk and Smidsrød, 1997; Smidsrød and Skjåk-Bræk, 1990). However, X. Wang et al. (2007) reviewing characteristics of alginate microspheres explained that alginate microparticles may be unstable in physiological environments as phosphate and citrate ions can extract Ca^{2+} from the alginate and liquefy the system. In addition, these authors raised concerns regarding the low mechanical strength of alginate drug delivery carriers. Similarly, starch microparticles have a disadvantage, as starch is susceptible to degradation in the gut (Heritage et al., 1996). Hence, these natural non-protein microparticles have to be stabilized often with synthetic polymers to be useful. For example, Heritage et al. (1996) grafted starch microparticles with a hydrophobic silicone polymer coating, 3-(triethoxysilyl)-propyl-terminated

polydimethylsiloxane (TS-PDMS) to effectively deliver orally human serum albumin (HSA). Therefore there is need to prepare microparticles from natural polymers which are stable in aqueous environment but still degradable. Microparticles made from kafirin may be suitable.

2.2 KAFIRIN

Kafirins are the aqueous alcohol soluble storage proteins (prolamins) of sorghum (Paulis and Wall, 1979) and located in protein bodies in the grain starchy endosperm (Taylor, Novellie and Liebenberg, 1985). Kafirins are very similar to zeins, the prolamin storage proteins of maize (DeRose, Ma, Kwon, Hasnain, Klassy and Hall, 1989) but are more hydrophobic (more strictly speaking, less hydrophilic) (reviewed by Duodu, Taylor, Belton and Hamaker, 2003; Belton, Delgadillo, Halford and Shewry, 2006). Four kafirin sub-classes have been identified as α-, β-, γ- and δ-kafirins (Shull, Watterson and Kirleis, 1991; Belton et al., 2006). Kafirins can be classified based on their differences in molecular weight, structure (Shull et al., 1991), cross-linking behaviour (El Nour, Peruffo and Curioni, 1998), amino acid composition and sequence (Belton et al., 2006; Mokrane, Lagrain, Gebruers, Courtin, Brijs, Proost and Delcour, 2009) (Table 2.1). From the hydration energy values, δ-kafirin is the most hydrophobic of the kafirin sub-classes. It is interesting to note from the hydration energies that γ-kafirin is more hydrophobic than α- and β-kafirins despite γ-kafirin being the only kafirin that is water soluble (Duodu et al., 2003). This may appear counterintuitive but Belton et al. (2006) explained that this is probably due to the high level of histidine in γ-kafirin (9 mols %) (De Freitas, Yunes, Da Silva, Arruda and Leite, 1994). As the pKa of histidine is about six (Edgcomb and Murphy, 2002), there may be a high degree of ionisation which leads to electrostatic repulsion and hence water solubility. The relative proportions of the different kafirin subclasses in total kafirin, which has been used by all workers on kafirin films and microparticles, are given in Table 2.1.

Hydrophobicity of a protein is important in regulating the release profile of encapsulated materials in the protein microspheres. For example, using soya protein isolate (SPI)/zein blend microspheres Chen and Subirade (2009) found that the encapsulated riboflavin release rate decreased progressively with increasing zein content. This was probably attributed to the resulting increased hydrophobicity due to zein proteins and hence decreased rate of hydration of the microspheres.

Table 2.1 Relative proportions of the different kafirin sub-classes in total kafirin

Kafirin	Molecular weight[a,b,c]	Proportion (%) of total kafirin[c,e]	Amino acid composition and sequence[a,b]	Hydration energy (kcal/ mol of 100 residues)[a]	Cross-linking behaviour[a, d]
α_1-	25–27k	66–84	Rich in non-polar amino acids, no Lys, one Trp, 10 blocks of repeated amino acids VIIPQXSLAPXAXXS	-144	Monomers oligomers and polymers
α_2-	22–24k			-133	
β-	16–20k	7–13	Rich in Met and Cys, two Trp LQMPGMGLQDLYGAGALMTM (M)GA (Q)X	-123	Monomers and polymers
γ-	28–31k	9–16	Rich in Pro, Cys, His. No Lys, Asn, Asp, Trp. Four repeats TLTTGGXGXQ	-100	Oligomers and polymers
	49k[*†]		Unknown		
δ-	13–15k		Rich in Met, no Lys, 1 Trp THIPGHLPLVM	-99	Unknown

[a]Belton et al. (2006); [b]Mokrane et al. (2009); [c]Shull et al. (1991); [d]El Nour et al. (1998); [e]Hamaker, Mohamed, Habben, Huang and Larkins (1995); [*]Evans, Schüssler and Taylor (1987); [†]Da Silva, Taylor and Taylor (2011)
X – Unidentified amino acid

2.2.1 Kafirin structure: physical and chemical properties

A study by Gao, Taylor, Wellner, Byaruhanga, Parker, Mills and Belton (2005) on native sorghum kafirins, using FTIR spectroscopy, showed a 58% α-helical conformation content. Similarly, working with both normal and mutant sorghums, Duodu, Tang, Wellner, Belton and Taylor (2001) found a 54–58% α-helical conformation content for uncooked sorghum kafirin. In addition, Wang et al. (2009) characterised the composition as well as chemical and physical properties of kafirin proteins in the distillers dried grains with solubles (DDGS), which is the main co-product from grain-based ethanol production. They extracted kafirin using three different methods: Acetic acid method (Taylor, Taylor, Dutton and De Kock, 2005a), HCl-ethanol method (Xu, Reddy and Yang, 2007) and NaOH-ethanol method (Emmambux and Taylor, 2003). The secondary structure of kafirin extracts from the three methods showed α-helical conformation dominance in all the three types of extracts with smaller proportions of β-sheet conformation. Therefore, it may be concluded that the native secondary structure of kafirin is rich in α-helical conformation.

However, when subjected to physical stress such as heat, the kafirin secondary structures generally become richer in β-sheet conformation as a result of unfolding (Duodu et al., 2001; Byaruhanga, Emmambux, Belton, Wellner, Ng and Taylor, 2006; Emmambux and Taylor, 2009). The pI of kafirin is 6 (Csonka, Murphy and Jones, 1926). This may be a useful parameter in manipulating the type of kafirin aggregation formed. This is because by adjusting the pH from above to below their isoelectric point the charge on proteins can be changed from negative to positive thereby resulting into different protein structures (reviewed by Van der Linden and Venema, 2007).

2.2.2 Kafirin digestibility

Kafirin has somewhat poor digestibility relative to other cereal prolamins, which worsens on wet cooking (reviewed by Duodu et al., 2003; Hamaker, Kirleis, Butler, Axtell and Mertz, 1987). The poor digestibility of wet-cooked kafirin is probably mainly due to disulphide cross-linking between the γ- and β- kafirin species, which inhibit the digestion of the major kafirin protein component, α-kafirin (Da Silva et al., 2011). As with microparticles prepared with poorly digestible zein (Parris et al., 2005; Hurtado-López and Murdan, 2006), the poor digestibility of kafirin may make kafirin microparticles more resistant to enzymatic degradation, enabling delivery of the encapsulated material to the desired site. A positive correlation has been shown between protein digestibility (under simulated gastric conditions) and the rate of release of antioxidant activity of catechin and condensed tannins encapsulated with kafirin microparticles (Taylor et al., 2009b).

2.2.3 Kafirin microparticles

As stated, kafirin microparticles, size 1–10 μm, as prepared by (Taylor et al., 2009a) using simple coacervation with acetic acid solvent are vacuolated. These kafirin microparticles produced high quality free-standing bioplastic films when about 11 times acetic acid as a proportion to kafirin content was used (Taylor et al., 2009c). In addition, these microparticles had the capacity to encapsulate bioactive compounds such as antioxidants (Taylor et al., 2009b), probably because their vacuolated structure provided larger binding surface area. However, as indicated the functionality of kafirin microparticles needs to be enhanced to broaden their potential applications.

2.3 MODIFICATION OF THE FUNCTIONAL QUALITY OF PROTEIN MICROPARTICLES

To prevent problems such as the fragility of protein microparticles and because of the short *in vivo* half-lives of bioactive compounds (time it takes for the bioactives to lose half their activity in a physiological environment), there is need to develop microparticles with improved functional quality such as physical strength and stability in aqueous environment (reviewed by Stark and Gross, 1994; Balmayor, Feichtinger, Azevedo, Van Griensven and Reis, 2009). Modification of proteins to enhance the functional properties of protein microparticles can be achieved by physical treatment (Patil, 2003; Vandelli, Romagnoli, Monti, Gozzi, Guerra, Rivasi and Forni, 2004), chemical cross-linking (Vandelli, Rivasi, Guerra, Forni and Arletti, 2001; Lee and Rosenberg, 1999), treatment with food components such as polyphenols (Xing, Cheng, Yang and Ma, 2004) and lipids (Önal and Landon, 2005), and enzymatic treatment (Dong, Xia, Hua, Hayat, Zhang and Xu, 2008). Such modifications can produce proteins with altered thermal stability, surface reactivity, lipophilicity, molecular weight, charge, shear stability and resistance to proteases (reviewed by Stark and Gross, 1994).

2.3.1 Physical treatment of protein microparticles

2.3.1.1 *Heat*

Heat has been applied to improve the functionality of protein microparticles. For example, Zhang and Zhong (2010) used thermal pre-treatment to enhance the heat stability of whey protein nanoparticles. This was aimed at averting the formation of aggregates or gels (at high protein concentrations e.g. >5%) when whey is heated above 60^{o}C. As deduced from this study heat treatment generally increased microparticles size through protein cross-linking, which results in protein polymerization. With certain protein microparticle preparation techniques, especially that involve particle micronization, an increase in temperature during microparticle preparation may result in a decrease in the size of protein microparticles. This was found by Bustami, Chan, Dehghani and Foster (2000) who used an Aerosol Solvent Extraction System (ASES) to generate microparticles from different types of proteins for aerosol delivery from aqueous-based solution. Their operating temperature was in the range 20–45°C. These authors found that at higher temperatures, the diameters of the microparticles were generally smaller compared to sizes obtained at relatively lower operating temperatures.

According to these authors, it seems that higher temperature during spray drying enhances mass transfer, and lowers density, both of which may induce a higher degree of super-saturation resulting in higher nucleation rate with correspondingly less particle agglomeration. Patil (2003) working with albumin microspheres explained that thermal stabilization occurs through direct reaction between functional groups on the albumin polypeptide side chains forming by inter-chain amide links (self-cross-linking), which renders the albumin insoluble. Thermal stabilization of albumin occurs at temperatures above 50^{o}C, although temperatures between 90–180^{o}C are commonly applied (Dubey, Parikh and Parikh, 2003). When Dubey et al. (2003) heat-stabilized albumin, there was a reduction in both particle size of the albumin microspheres and their drug entrapment efficiency for 5-fluorouracil (5-FU), an anticancer drug, with increasing temperature and time. This finding indicated that modification of protein microparticles by conventional heat treatment might not be an ideal method for heat sensitive encapsulated active ingredients. Microwave cross-linking has also been explored as a means of improving the loading and the drug release properties of gelatin microspheres loaded with diclofenac, a non-steroidal anti-inflammatory agent (Vandelli et al., 2004). This heat treatment enhanced the loading capacity and protection of the drug from degradation.

2.3.1.2 *Application of mechanical stress*

During the preparation of protein microparticles stress may be introduced through, for example, mechanical shearing and/or agitation or through exposure to ultrasound (reviewed by Bilati, Allemann and Doelker, 2005). Generally, smaller protein microparticles are formed by increased speed and longer mixing times due to the formation of smaller emulsion droplets (Patil, 2003; Whittlesey and Shea, 2004; Freitas et al., 2005), which result from stronger shear forces and increased turbulence. A study of kafirin microparticles prepared using aqueous ethanol as the solvent showed an increase the size of holes (vacuoles) in the microparticles when higher shear was applied during mixing (Taylor et al., 2009a). As explained by these authors, it seems that the increased turbulence with high shear would incorporate more air into the liquid phase, thereby creating bubbles. Then small bubbles would possibly coalesce to form larger bubbles before being entrapped within the microparticles as the protein precipitates. However, in the same study, it was observed that kafirin microparticles prepared using a different solvent (glacial acetic acid), lost their spherical structure instead forming a continuous matrix apparently being broken into

fragments, which aggregated together. This study highlighted the necessity of choosing an appropriate solvent for preparation of microparticles for particular applications. It also underscored the need to explore possibilities of including additives such as porogens in certain solvent systems if needed to improve the porosity of protein microparticles. A porogen is a soluble additive included during microparticle preparation, which after microparticle formation is leached out, generating a highly porous matrix (Lee, Soles, Vogt, Liu, Wu, Lin, Kim, Lee, Volksen and Miller, 2008). Nonetheless, agitation can be used to manipulate vacuole sizes of protein microparticles thereby increasing their encapsulation capacities (Taylor et al., 2009a). As discussed, stirring to create a flow instead of turbulence can be used to enhance formation of fibrils (Bolder, Sagis, Venema and Van der Linden, 2007). This shows that regulation of the amount of stress in the system can be used to manipulate the structure of the protein microparticles to suit specific applications.

2.3.1.3 *Manipulation of acid concentration*

Increasing the concentration of an acid used in the preparation of protein microparticles has been shown to result in larger kafirin microparticles with a less continuous structure (Taylor et al., 2009a), possibly due to aggregation of protein molecules through denaturation. As a possible cause of protein aggregation, these authors suggested that higher acid concentration may unfold the kafirin secondary structure from a predominately α-helical structure to β-sheet conformation. As the β-sheet conformation is more mobile from a molecular perspective, this would expose previously hidden hydrophobic amino acids allowing hydrophobic interactions between polypeptide chains. The increase in molecular mobility due to the β-sheet conformation would also expose other ionic and neutral amino acids which would then be available to form inter- and intra- molecular hydrogen bonds. The combination of hydrogen bonding and hydrophobic interaction between polypeptide chains would then result in protein aggregation. Similarly, working with SPI-zein composite, Chen and Subirade (2009) noted that swelling of microspheres was minimal near the isoelectric point (pI) and greater at lower pH (pH 1.2) or higher pH (pH 7.4), which probably indicated that swelling behaviour is governed mainly by the net charge of the protein molecules. As discussed, near the pI the numbers of charged groups on the polypeptide chains ($-NH_3^+$ and $-COO^-$) are presumed to be nearly equal, and few ionized groups are free to repel each other (Krebs et al., 2007). At lower pH, the chains bear a strong positive net charge due to $-NH_3^+$ groups, whereas at higher pH, they bear a strong negative net charge because of $-COO^-$ groups. The

resulting electrostatic repulsion allows the solvent medium to diffuse into the protein network causing more swelling and larger microspheres. Therefore, during preparation, the pH of the system can be manipulated to form microparticles with the required size and structure for particular applications.

2.3.2 Cross-linking proteins with enzymes

The stability of a protein can be improved by cross-linking the protein prior to preparation of microparticles by phase separation process by addition of an enzyme, which catalyses intra- and/or inter-molecular cross-linking of the protein, such as transglutaminase, or protein disulphide isomerase (Mathiowitz, Bernstein, Morrel and Schwaller, 1993). Transglutaminase (protein-glutamine:amine γ-glutamyl transferase) catalyses an acyl transfer reaction in which carboxyamide groups of peptide-bound glutamine residues are the acyl donors and a variety of primary amines are the acyl acceptors (Figure 2.2a) (Ohtsuka, Sawa, Kawabata, Nio and Motoki, 2000).

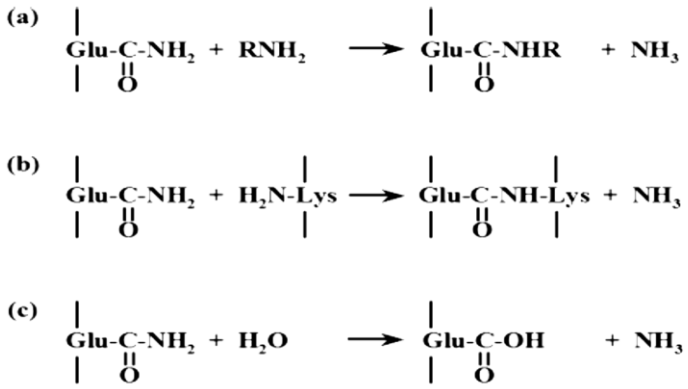

Figure 2.2 Reactions catalysed by transglutaminase. (**a**) Acyl-transfer reaction; (**b**) Cross-linking reaction between glutamine and lysine residues of proteins or peptides. The resultant bridge is called ε-(γ-glutamyl)-lysine bond; (**c**) Deamidation (Yokoyama et al., 2004).

When ε-amino groups of peptide-bound lysine residues are the acceptors, protein cross-linking occurs through ε-(γ-glutamyl)-lysine bridges (Figure 2.2b). In the absence of available amines, the enzyme may also catalyse the hydrolysis of glutamine residues to glutamate residues during which water molecules are used as acyl acceptors (Figure 2.2c) (Yokoyama, Nio and Kikuchi, 2004; Motoki and Seguro, 1998). The action of this enzyme leads to the post-translational modification of proteins through either the formation of intra- and intermolecular isopeptide bonds or the covalent attachment of amines such as polyamine

and putrescine (Ikura, Goto, Yoshikawa, Sasaki and Chiba, 1984). The large polymers so formed may be chemically and enzymatically resistant and mechanically strong. Transglutaminase has been widely applied commercially in the food industry in protein cross-linking reactions (reviewed by Lantto, 2007). Enzyme concentration may be an important parameter especially when enzymatic modification is used to enhance the functionality of protein microparticles. An example is demonstrated in the work of Dong et al. (2008) on transglutaminase-hardened gelatin-gum arabic spherical multinuclear microcapsules (SMMs) prepared by complex coacervation. These authors found that lower concentrations of transglutaminase led to lower structural stability in the resulting SMMs. This was probably because decreasing the transglutaminase concentration results in lower enzymatic activity per volume and thus possibly lower microcapsule cross-linking density. Therefore, the microcapsule hardening ability becomes poor, and the resulting structure disintegrates easily.

Sometimes protein microparticles are preferred with lower molar mass. For example, peptides of gluten of molar mass <10 kDa, preferably 3–5 kDa may be required due to their water-retaining capacity and their conditioning effect (reviewed by Auriol, Paul and Monsan, 1996). As stated by these authors, these low molecular weight peptides can be obtained through enzymatic hydrolysis with proteases such as Alcalase (Novozymes), pepsin or acid protease from *Aspergillus niger*. The peptides so derived have marked hydrophobic characteristics. The functionality, surface properties and molecular weight distribution of the protein can also be modified by hydrolysis with other proteases, such as papain or chymotrypsin, to yield peptides having similar solubility characteristics as the untreated protein (Haralampu, Sands and Gross, 1993).

2.3.3 Cross-linking protein microparticles with chemicals

Chemical cross-linking procedures have been applied to overcome the problem of rapid solubilisation of protein microparticles in aqueous environments, which results in fast drug release profiles (Digenis, Gold and Shah, 1994). Furthermore, as hydrophilic polymeric system, protein microparticles have difficulties achieving sustained drug release as when the system absorbs water and swells, drugs will rapidly diffuse out (reviewed by Latha, Rathinam, Mohanan and Jayakrishnan, 1995). The chemicals commonly used to cross-link protein microparticles for microparticle stability in aqueous environment include aldehydes such as glutaraldehyde (Lee and Rosenberg, 1999, 2000), formaldehyde and glyceraldehyde (Vandelli et al., 2001). Cross-linking of protein with an aldehyde involves the reaction of free

amino groups of peptide chains with the aldehyde (carbonyl) groups (Bigi, Cojazzi, Panzavolta, Roveri and Rubini, 2002) (Figure 2.3). The carbonyl groups of the aldehydes can react with any nitrogen in a protein (Kiernan, 2000). Intra- and intermolecular links are formed that could connect atoms of neighbouring but not interacting molecules, yielding artificial protein oligomers that lack biological significance (reviewed by Fadouloglou, Kokkinidis and Glykos, 2008).

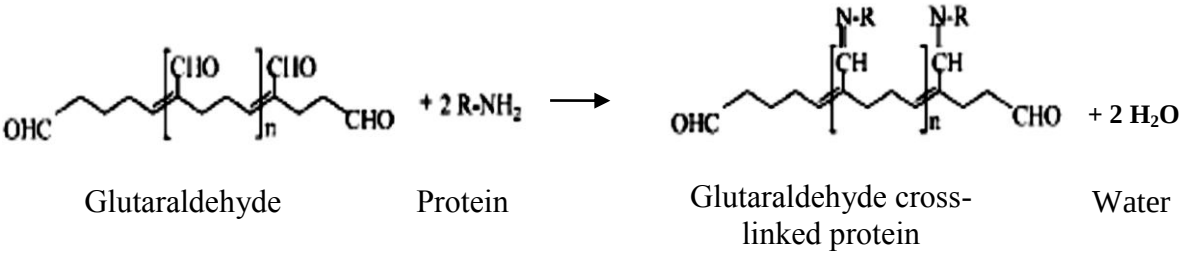

Figure 2.3 Cross-linking reaction between glutaraldehyde and protein (adapted from Migneault, Dartiguenave, Bertrand and Waldron, 2004; Kiernan, 2000).

Glutaraldehyde is the most efficient among these three aldehydes in cross-linking of food proteins (reviewed by Gerrard and Brown, 2002). This is probably due to the presence of dicarbonyl groups in a glutaraldehyde molecule, which provide two reactive moieties (Gerrard and Brown, 2002) and the fact that it has five-carbon species considered optimal for protein cross-linking reactions (reviewed by Meade, Miller and Gerrard, 2003). According to Migneault et al. (2004), it is the polymeric forms of glutaraldehyde that are involved in the cross-linking reaction. The core release properties of a protein microparticle can substantially be improved by cross-linking protein with glutaraldehyde. For example, to improve the efficiency of encapsulation of theophylline (a water-soluble drug used in therapy for respiratory diseases such as asthma) with whey microparticles, Lee and Rosenberg (1999) cross-linked the whey protein with glutaraldehyde-saturated toluene. This great improvement in the encapsulation efficiency through cross-linking was probably due to creation of a dense microparticle wall matrix occasioned by the formation of whey protein-glutaraldehyde-saturated toluene meshwork, which impeded diffusion of the microencapsulated theophylline molecules. In addition, Vandelli et al. (2001) working with gelatin microspheres loaded with clonidine hydrochloride (an antihypertension drug), found that cross-linking with D,L-glyceraldehyde resulted in a more gradual and sustained systolic blood pressure (SBP) reduction and the antihypertensive effect than uncross-linked microspheres. A main disadvantage of using chemical cross-linking as opposed to heat or transglutaminase cross-

linking is the toxicity of the residuals of chemical cross-linking agents and the *in vivo* biodegradation products of the chemical cross-linked macromolecule (reviewed by Vandelli et al., 2004).

2.3.4 Cross-linking with polymeric food components

Tannins are plant polyphenols with an exceptional ability to precipitate protein (Emmambux and Taylor, 2003; Charlton, Baxter, Khan, Moir, Haslam, Davies and Williamson, 2002). Xing et al. (2004) studied the influence of the tannins on the morphology and structures of the gelatin-acacia complex microcapsules used to microencapsulate capsaicin (the main pungent ingredient in hot peppers). These authors found that when compared to the use of glutaraldehyde, tannin cross-linked microcapsules had relatively high encapsulation efficiency and relatively high drug content with a good dispersion and an even distribution and shape. Similarly, the ability of polyphenols to inhibit degradation of protein microparticles was reported by Taylor et al. (2009b) who found little degradation of the kafirin microparticles encapsulating sorghum condensed tannin and catechin while still releasing reasonably good antioxidant activity. This was probably a result of synergistic actions of hydrogen bonding and hydrophobic effects when tannins interact with proteins (Kawamoto, Mizutani and Nakatsubo, 1997). Studies have shown an intrinsic complexity of the molecular recognition processes that occur between tannins and proteins such as gelatin (Madhan, Muralidharan and Jayakumar, 2002).

The performance of protein microparticles for delivery of bioactives can also be improved by using food compounds. For example, by adding different types of lipids, Önal and Landon (2005) found a substantial improvement in quality of zein microparticles, measured by encapsulation, delivery and retention efficiencies of riboflavin in early fish larvae. These workers did not provide a clear explanation why this was so. However, the introduction of lipid in to the system may have induced protein-lipid interaction.

2.4 PROTEIN MICROPARTICLE FILMS

These are bioplastic films prepared from protein microparticles. There are two process pathways used for producing bioplastic protein films, the dry (melt process) and the wet process (reviewed by Cuq, Gontard and Guilbert, 1998; Zhang and Mittal, 2010). The dry process uses a thermal or thermo-mechanical process based on thermoplastic properties of

biopolymers when plasticized and heated above their glass transition temperatures under low water content. For example, Lai, Padua and Wei (1997) used the dry process to prepare 0.5 mm thick zein films (sheets) plasticized with palmitic and stearic acids. In the wet process (solvent process) or also known as casting, the protein microparticles are typically dissolved or dispersed in suitable solvents with plasticizers. The resulting solutions or dispersions form free-standing films or coatings on a plate by casting the mixture and subsequent drying. This procedure is generally used for pre-formed films and coatings (reviewed by Cuq et al., 1998). Casting is the most often used protein film-forming method (reviewed by Zhang and Mittal, 2010). These protein films in general are water sensitive and lack mechanical strength but are good oxygen barriers (reviewed by Cuq et al., 1998; Zhang and Mittal, 2010).

Studies have shown that films produced from protein microparticles have superior functional quality than conventional protein films. For example, Taylor et al. (2009c) found that kafirin microparticle films had smoother film surface and lower water vapour permeability than the conventional cast films from kafirin. Likewise, Cook and Shulman (1998) working with zein found that zein microparticle films had no voids or porosity in contrast to conventional zein films, which had large void spaces as assessed by scanning electron microscopy (SEM). Researchers have shown that protein microparticle films have potential for useful novel applications. For example, Dong, Sun and Wang (2004) prepared zein microparticle films onto which human liver cells and mice fibroblast cells could attach and proliferate. Similarly, in a study by Wang, Lin, Liu, Sheng and Wang (2005), they reported a heparin-loaded zein microsphere films with potential for application as drug-eluting coating films used for cardiovascular devices such as stents.

2.5 APPLICATION OF PROTEIN MICROPARTICLES

The majority of applications of protein microparticles is in the medical field, especially as carriers for drug delivery (reviewed by Haidar, Hamdy and Tabrizian, 2009; Tan et al., 2010). However, the boundaries between food, medicine and cosmetics are already obscure and the advent of nanoparticles that can interact with biological entities at a near-molecular level is likely to blur further these boundaries (reviewed by Chaudhry et al., 2008). Some food and cosmetic companies are collaborating to develop cosmetic nutritional supplements. For example, in 2002, L'Oréal and Nestlé formed a joint venture, Laboratoires Innéov (Charles, 2002). Innéov's first product, called "Innéov Firmness," contains lycopene (Marketing Week, 2003). Applications of protein microparticles in the food industry include delivery of

bioactive compounds or agents for development of improved flavour, colour, texture and consistency of foodstuffs, increased absorption and bioavailability of nutrients and health supplements (reviewed by Jones and McClements, 2010; Livney, 2010; Schrooyen et al., 2001). Table 2.2 highlights representative bioactive agents delivered by protein microparticles/nanoparticles. Protein microparticles can also be used as new food packaging materials with improved mechanical, barrier and antimicrobial properties, and nano-sensors for traceability and monitoring the condition of food during transport and storage (reviewed by Chaudhry et al., 2008).

In the patent of Stark and Gross (1991), they claim that their microparticles, with size 0.1–4.0 µm, can be used as a fat substitute in food. Protein microparticles could increase the survival of healthy probiotic bacteria during their transit through the gastrointestinal tract by protecting bacteria in both the carrier food and in the human stomach (Marteau et al., 1997; Picot and Lacroix, 2004) with potential health benefits. Protein microparticles have potential for application in the biomedical sector in tissue engineering (Gong et al., 2006) as scaffolds or sutures, carrier for bioactive compounds such as bone morphogenetic proteins (Haidar et al., 2009). Biomaterials applied as carriers for controlled delivery of bioactives offer many advantages over the inorganic systems such as fullerene, gold, silver and silica. Some of the advantages of biomaterials include the increase of treatment effectiveness and significant reduction of toxicity, due to their biodegradability property (reviewed by Xu et al., 2011).

Table 2.2 Examples of bioactive compounds or agents delivered by protein microparticles

Bioactive category	Bioactive agent	Protein
Nutraceuticals	*Vitamins:*	
	D (Semo, Kesselman, Danino and Livney, 2007)	Casein
	B1 (Benichou, Aserin and Garti, 2007)	Whey protein isolate
	riboflavin (Chen and Subirade, 2009)	Soy-Zein blend
	Minerals:	
	Fe^{2+} (Sugiarto, Ye and Singh, 2009)	Casein/ Whey protein isolate
	Mg^{2+} (Bonnet, Cansell, Berkaoui, Ropers, Anton, and Leal-Calderon, 2009)	Casein
	Omega 3 oils and other health promoting fatty acids:	
	fish oil (Patten, Augustin, Sanguansri, Head and Abeywardena, 2009)	Casein
	flax oil (Quispe-Condori, Saldaña and Temelli, 2011)	Zein
	docosahexaenoic acid (DHA) (Zimet and Livney, 2009)	β-lactoglobulin
	Essential oils:	
	oregano, cassia and red thyme (Parris et al., 2005)	Zein
	Carotenoids:	
	β-carotene (López-Rubio and Lagaron, 2012)	Whey protein concentrate
	lycopene (Gouranton, El Yazidi, Cardinault, Amiot, Borel and Landrier, 2008)	Bovine serum albumin
	Polyphenols:	
	resveratrol (Bharali, Siddiqui, Adhami, Chamcheu, Aldahmash, Mukhtar and Mousa, 2011)	Bovine serum albumin
	condensed tannins and catechin (Taylor et al., 2009b)	Kafirin
	epigallocatechin gallate (EGCG) (Shutava, Balkundi and Lvov, 2009)	Gelatin
Probiotics and other microorganisms	Bifidobacteria (Picot and Lacroix, 2004)	Whey protein
	recombinant yeasts (Hebrard, Blanquet, Beyssac, Remondetto, Subirade and Alric, 2006)	Whey protein

Bioactive category	Bioactive agent	Protein
Therapeutics	*Anti-microbial:*	
	nisin and thymol (Xiao, Davidson and Zhong, 2011)	Zein
	Parasiticide (endectocide):	
	ivermectin (Gong, Sun, Sun, Wang, Liu and Liu, 2011; Liu et al., 2005)	Zein
	Cancer therapy:	
	paclitaxel (Gong, Huo, Zhou, Zhang, Peng, Yu, Zhang and Li, 2009)	Bovine serum albumin
	5-fluorouracil (Maghsoudi, Shojaosadati and Farahani, 2008)	Bovine serum albumin
	Anti-diabetic drug:	
	metformin (Xu, Jiang, Reddy and Yang, 2011)	Zein
	Peptidic-drugs:	
	bone morphogenetic protein-2 (Bessa, Balmayor, Hartinger, Zanoni, Dopler, Meinl, Banerjee, Casal, Redl, Reis and Van Griensven, 2010)	Silk fibroin
	Other drugs:	
	theophylline (asthma drug) (Latha et al., 1995)	Casein
Enzymes	bile salt-hydrolase (Lambert, Weinbreck and Kleerebezem, 2008)	Whey protein
Flavours/aromas/fragrances	ethyl hexanoate (Giroux and Britten, 2011)	Whey protein

2.6 TECHNIQUES FOR STUDYING THE STRUCTURE OF PROTEIN MICROPARTICLES

The techniques used to study the structure of protein microparticles depend on the information required. Some of the common techniques are microscopy, spectroscopy, electrophoresis, and differential scanning calorimetry (DSC). Often these techniques are used in combination for verification. The more important methods will be discussed briefly.

2.6.1 Microscopy

Microscopy is an important technique for visualization in the study of protein and protein products structure. Yang, Wang, Lai, An, Li and Chen (2007) provides a summary of some of the common microscopy techniques that are used in studying the structure of protein microparticles. The use of a number of different imaging techniques is recommended in order to compare and confirm results. The main features of some the microscopy techniques and protein microparticle type applications are shown in Table 2.3.

2.6.2 Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy provides information about the secondary structure composition of proteins. Each protein has a characteristic set of absorption bands in its infrared spectrum. Typical bands found in the infrared spectra of proteins and polypeptides include the Amide I and Amide II, which arise from the amide bonds that link the amino acids (reviewed by Pelton and McLean, 2000; Surewicz, Mantsch, and Chapman, 1993). The Amide I band is due mainly to C═O stretch (80%) and weak N–H bend, and the stretching of the C−N bond, while Amide II band is due to N–H bend (60%) and C–N stretch (40%). Conformational sensitivity of amide bands are mainly influenced by H-bonding and the coupling between transition dipoles (reviewed by Pelton and McLean, 2000). In kafirin, the Amide I band occurs at wave number ≈1650–1620 cm^{-1} while Amide II band occurs in the ≈1550–1500 cm^{-1} region (Duodu et al., 2001). FTIR has been used by many workers to determine the secondary structure of protein microparticles and in turn relate the findings to the functionality of the protein microparticles.

Table 2.3 Characteristics of different microscopy techniques and their application in protein microparticle type research (adapted from Yang et al., 2007)

Microscopy	Advantages	Disadvantages	Examples of application
Light microscopy	Large scan area; Fast scan speed; Cheap	Only 2D; Need pre-treatment; Low resolution and magnification	Kafirin microparticle films (Taylor et al., 2009c); Microwave-treated gelatin microspheres (Vandelli et al., 2004)
Scanning electron microscopy (SEM)	High resolution; Fast scan speed	Only 2D; Need pre-treatment; Not native status	Zein films (Lai and Padua, 1997), zein scaffolds (Gong et al., 2006), zein microspheres (Suzuki, Sato, Matsuda, Tada, Unno and Kato, 1989; Parris et al., 2005); whey protein based microcapsules (Lee and Rosenberg, 1999)
Transmission electron microscopy (TEM)	Nanoscale; High resolution; Fast scan speed	Only 2D; Need pre-treatment; Not native status	Ovalbumin nanospheres (Coombes et al., 2001); Whey protein beads (Beaulieu, Savoie, Paquin and Subirade, 2002); Kafirin microparticles (Taylor et al., 2009a)
Confocal laser scanning microscopy (CLSM)	Study dynamic process; Fast scan speed; 2D and 3D; In situ	Limited excitation wavelengths available with common lasers, occurring over narrow bands and are expensive to produce in the ultraviolet region; Need pre-treatment	Silk fibroin microspheres (X. Wang, Wenk, Matsumoto, Meinel, Li and Kaplan, 2007)
Atomic force microscopy (AFM)	High resolution (nanoscale); Minimal sample preparation; 2D and 3D; In air or liquid, in situ, continuous process; Can be manipulated	Small scan size; Slower scan speed; Difficult for soft material	Zein films (Guo, Liu, An, Li and Hu, 2005), Zein globules (Gao, Ding, Zhang, Shi, Yuan, Wei and Chen, 2007), Zein microparticles (Wang et al., 2008), Gliadins (McMaster, Miles, Kasarda, Shewry and Tatham, 2000).

For example, Taylor et al. (2009a) working on kafirin microparticles used FTIR with which they found that formation of kafirin microparticles was associated with β-sheet conformation at the expense of α-helical conformation. Similarly, Chen and Subirade (2009) working with riboflavin as the model drug used FTIR to characterise SPI, zein, and SPI/zein blends as potential delivery systems for nutraceutical products. Other workers who have used FTIR to study protein microparticles include López-Rubio and Lagaron (2012) working on whey protein capsules obtained through electrospraying for the encapsulation of β-carotene. Most studies on protein microparticles with FTIR use the Amide I band determination of protein secondary structure rather than the amide II band. This is probably because Amide I band mainly arises from only one of the amide functional groups, in contrast to the Amide II mode (Jackson and Mantsch, 1995).

2.6.3 Electrophoresis

This is a technique that separates proteins under the influence of an electric field, through a sieving medium, usually a polyacrylamide gel (reviewed by Yada, Jackman, Smith and Marangoni, 1996). The proteins are separated based on their differences in molecular size and/or charge. A common method, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) separates protein molecules based on their sizes. SDS-PAGE has been used often in determination of molecular weight distribution in many studies of protein microparticles. For example, Parris et al. (2005) used SDS-PAGE to measure the rate of zein nanosphere digestion thereby determining the rate of release of essential oils encapsulated by zein nanospheres. Taylor et al. (2009a) working on kafirin microparticles used SDS-PAGE to show that despite evidence of aggregation of the kafirin proteins during microparticle formation, there was no significant polymerisation, in contrast to what happens when kafirin is heated under moist conditions. In a related study, these workers used SDS-PAGE to show little enzymatic degradation of the kafirin microparticles encapsulating sorghum condensed tannin, which contrasted the almost complete degradation of kafirin microparticles not encapsulating the tannins (Taylor et al., 2009c). Zhang, Luo and Wang (2011) used SDS-PAGE to determine the stability of zein microparticles in mild acidity (pH 6.5) where they found that zein retained its primary structure upon the mild pH treatment.

2.7 CONCLUSIONS

Protein microparticles have potential for use in preparation of useful biomaterials. However compared to similar products prepared using synthetic materials, protein biomaterials have shortcomings such as poor mechanical properties, poor water stability and relatively lower binding capacity for bioactive compounds. These limitations must be addressed to realize the full potential of protein microparticles. This could be achieved by modifying the properties of the protein microparticles, and hence improving their functional properties, which will in turn increase their end use application. Modification of the protein microparticles can be achieved by physical, chemical and enzymatic cross-linking or a combination of these treatments. These treatments are usually performed on the proteins prior to or during preparation of the microparticles. An alternative approach that is not often applied is treatment of the protein microparticles after their preparation. The relatively high hydrophobicity and slow enzymatic hydrolysis of sorghum kafirin and the fact kafirin can be used to produce vacuolated microparticles, provides an opportunity to produce high quality microparticles that have potentially many applications in the food and pharmaceutical industry as well as the biomedical field. However, to make them more useful as biomaterials it is important to increase the size of the kafirin microparticles to suit applications where large structures with a high degree of interconnected porosity are required. In addition, it is also necessary to improve both the tensile properties and water resistance of the bioplastic films prepared from the kafirin microparticles in order to make them applicable in aqueous conditions.

3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

a. Treating kafirin microparticles with heat, transglutaminase and glutaraldehyde will change the morphology of the microparticles. These microparticles will be larger and more inert as a result of kafirin protein polymerization. Heat treatment has been shown to result in kafirin polymerization as a result of disulphide cross-linking of kafirin proteins (Emmambux and Taylor, 2009; Duodu et al., 2003). Despite the fact that kafirin has very low lysine content (Belton et al., 2006), in the absence of lysine, transglutaminase catalysed reactions should proceed through deamidation, which is a secondary reaction pathway, where kafirin glutamine in converted to glutamic acid (Yokoyama et al., 2004). Glutaraldehyde cross-linking occurs through the formation of non-disulphide bonding between the carbonyl groups (C═O) of the aldehyde and free amino groups ($-NH_2$) of the protein amino acid (Farris, Song and Huang, 2010).

b. Modification of kafirin microparticles by heat, transglutaminase and glutaraldehyde will result in an improvement in the quality of products prepared from kafirin microparticles such as bioplastic films and delivery devices for bioactive compounds. This is because of kafirin protein polymerization. It has been shown that protein polymerization by heat, transglutaminase or glutaraldehyde slows down the rate of release of bioactive compounds bound by protein microparticles (Prata, Zanin, Ré and Grosso, 2008) and improves protein film water stability and related properties (Byaruhanga, Erasmus and Taylor, 2005; Chambi and Grosso, 2006; Sessa, Mohamed, Byars, Hamaker and Selling, 2007), probably as result of increase in the protein hydrophobicity (Reddy, Tan, Li and Yang, 2008). By slowing down rate of release of bound bioactive compounds as result of increased kafirin hydrophobicity, the kafirin microparticle-bioactive system will be more efficacious. The efficacy of a bioactive compound is achieved by maintaining its concentration at the target site for a sufficient period of time (Bessa, Casal and Reis, 2008).

c. The kafirin microparticle-bioactive compound complex will be a safe biomaterial system as kafirin is non-allergenic (Ciacci, Maiuri, Caporaso, Bucci, Del Giudice, Massardo, Pontieri, Di Fonzo, Bean, Ioerger and Londei, 2007) due to the fact that it does not contain any of the protein amino acid sequences known to be toxic to coeliacs (reviewed

by Wieser and Koehler, 2008). As explained by these authors, protein toxicity is associated with repeating amino acid sequences such as QPQPFPPQQPYP and (Q) QPQQPFP, which are absent in kafirin proteins.

3.2 OBJECTIVES

The overall objective of this study is to improve the quality of kafirin microparticles in order to expand their potential application.

Specific objectives:

1. To determine the effects of modification with heat, transglutaminase and glutaraldehyde on the functionality of kafirin microparticles with respect to their size, structure and chemical properties.
2. To determine the effects of modification of kafirin microparticles on the quality of products prepared from them such as bioplastic films and binding devices for bioactive molecules such as bone morphogenetic proteins (BMPs).

To establish the safety, biodegradability and efficacy of kafirin microparticle-bioactive system.