Chapter 1:

General Introduction and Objectives of the Study

1.1. Pellets as solid dosage forms

Generally, multi-particulate formulations or multiple unit-dosage forms (pellets, microtablets and granules) are continuously produced since they permit flexibility in development. Pellets, being multiple unit-dosage forms, are widely used as they offer both manufacturing and therapeutic advantages over single-unit solid dosage forms (Dukić-Ott et al., 2009).

As defined by Ghebre-Sellassie, (1989); pellets are spherical, free-flowing granules with a narrow size distribution, typically varying between 500 and 1500 µm in size for pharmaceutical applications. They are formed as a result of a pelletization process which is an agglomeration process that converts fine powders or granules of bulk drugs and excipients into small, free-flowing, spherical or semi-spherical units. There are different techniques applicable for the production of pellets in pharmaceutical industries which are:

(i) Solution and suspension layering. This process uses conventional coating pan or fluidized bed with conventional top spray or Wurster bottom spray to apply drug/binder solution or suspension to solid cores that can be inert materials or granules of the same drug.

(ii) Dry powder layering. This involves the use of rotor-granulators/rotor tangential spray fluid-bed to deposit successive layers of dry powder (drug and excipients) on inert materials with the help of an adhesive solution/binding liquid.
(iii) **Direct powder pelletisation.** The technique uses high shear mixers and centrifugal fluid-bed or rotary fluid-bed granulators to apply agglomeration liquid direct to a powder mixture of a drug and expients followed by pelletization by means of a rotating disc. A binder can be added as a liquid (wet pelletization) or added as a molten binder before or during the process (melt pelletization).

(iv) **Extrusion-Spheronisation process.** This is the most employed technique as it offers the advantage to incorporate high amounts of active pharmaceutical ingredient, without producing an excessively large particle of drug-loaded pellets apart from being more efficient than the other techniques for producing pellets.

Extrusion can be defined as the process of forcing a material through an orifice or die under controlled conditions thus forming cylinders or strands called extrudates. During spheronization, these extrudates are broken into small cylinders and consequently rounded into spheres (pellets). Hence, extrusion/spheronization is a multiple-step process capable of making uniformly sized spherical particles referred to as pellets and involving the following sequential steps: (1) dry blending, (2) wet granulation, (3) extrusion, (4) spheronization, (5) drying, and (6) optional screening (Erkoboni, 2003).

**Figure 1.1:** Extrusion/spheronization process flow chart with individual processing variables.
The end product from each of the steps is shown Figure 2.

![Figure 1.2: Product produced by the first four extrusion/spheronization process steps: (a) Powder from dry mixing, (b) granules from granulation, (c) extrudate from extrusion, and (d) spheres (pellets) from spheronization.](http://www.pharmamanufacturing.com/articles/2007/080.html)

**1.2. Mechanisms of pellet formation**

The transformation from cylinder-shaped extrudate to a sphere occurs in various stages. Two models have been proposed to describe the mechanism as shown graphically in Figure 1.3.

![Figure 1.3: Pellet-forming mechanisms by spheronization process.](http://www.pharmamanufacturing.com/articles/2007/080.html)
One model was proposed by Baert and Remon (1993) who suggested that, the initial cylindrical particles (Fig.1.3: 1a) are deformed into a bent, rope-shaped particle (Fig.1.3: 1b), then form a dumbbell with a twisted middle (Fig.1.3: 1c). The twisting action causes the dumbbell to break into two spherical particles, with a flat side having a hollow cavity (Fig.1.3: 1d). Continued action in the spheronizer causes the particles to round off into spheres (Fig.1.3: 1e).

The second model proposed by Rowe (1985), describes a transition whereby the cylindrical particles (Fig.1.3: 2a) are first rounded off into cylindrical particles with rounded edges (Fig.1.3: 2b), then form dumbbell-shaped particles (Fig.1.3: 2c), ellipsoids (Fig.1.3: 2d), and finally, spheres (Fig.1.3: 2e) (Erkoboni, 2003).

1.3. Pellet properties

The properties of pellets are listed in Table 1.1 together with different evaluation methods (Knop and Kleinebudde, 2005; Trivedi et al. 2007).

<table>
<thead>
<tr>
<th>Table 1.1: Properties of pellets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Properties</td>
</tr>
<tr>
<td>Particle shape, sphericity</td>
</tr>
<tr>
<td>Surface texture/roughness</td>
</tr>
<tr>
<td>Mean particle size</td>
</tr>
<tr>
<td>Particle size distribution</td>
</tr>
<tr>
<td>Porosity</td>
</tr>
<tr>
<td>Density</td>
</tr>
<tr>
<td>Mechanical strength of pellets</td>
</tr>
<tr>
<td>Flow properties</td>
</tr>
<tr>
<td>Drug content</td>
</tr>
</tbody>
</table>
1.4. Advantages of pellets

Pellets offer several advantages as multiple unit dosage forms:

- The gastrointestinal transit time, especially the residence time in the stomach, is more uniform and less sensitive to food ingestion compared to single unit dosage forms because the small pellets can pass the pylorus even in the closed state. This leads to reduced variability in drug plasma absorption profiles between subjects and within the same patient, as a result of even distribution in the GI tract.
- The particles are well dispersed after swallowing and hence the local concentration of drug is relatively low and irritation of the gastrointestinal mucosa is therefore less.
- Pellets with a coating for modified release have a lower risk of dose dumping than coated tablets. That is, immediate release of API from a sustained release single unit tablet with a defective coating is very much reduced in pellets.
- Pellets with different coatings can be mixed and filled into capsules to enable the desired dissolution profile to be achieved.
- Pellets with incompatible bioactive drugs can be coated and filled into capsules or compressed into tablets without the risk of interaction of the substances during preparation and storage so that can be administered as a single unit dosage form.
- They can be formulated as sustained, controlled, or site-specific delivery of the drug from coated pellets.
- The manufacturing advantages include: - ease of capsule filling because of improved flow, reduced friability, narrow particle size distribution, ease of coating and uniform packing (Vervaet, et al., 1995; Knop and Kleinebudde., 2005; Trivedi et al., 2007).
1.5. Pellet applications

Potential applications of pellets are many and can be categorized into pharmaceutical, food and agricultural uses as listed below (www.lcicorp.com.) Pellets can be formulated into controlled release pellets, multi particulate systems, and enteric drugs or developed as novel drug delivery systems as they permit flexibility in development (Figure 1.4).

**Pharmaceutical application of pellets**

![Flow chart for pharmaceutical applications of pellets](image)

**Figure 1.4:** Flow chart for pharmaceutical applications of pellets

**Food applications of pellets:** Pellets can be formulated for food application purposes as sugars and sweeteners, catalysts or beverage preservatives.

**Agriculture applications of pellets:** In agricultural business, pellets can be formulated as fish foods and also into fertilizer as agricultural chemicals.
1.6. Process benefits of extrusion/spheronization

Pellets are widely produced by extrusion-spheronization process in pharmaceutical industries as the technology offers the following benefits: ease of operation and scale-up, high yield with low wastage, better and narrower particle size distribution, improved flowability, production of pellets with low friability, production of pellets that are suited for easy film coating, production of uniformly-sized distributed pellets, production of wide variety of engineered controlled-release drugs, reproducible packing and low dust generation.

1.7. Objectives of the study

It is with regard to these resourceful uses that this study identified two areas of research, based on a food and a pharmaceutical application of pellets as delivery system with the following objectives:

1. To develop a multiparticulate formulation containing stabilizing agents for improved colloidal stability in the beer brewing process, via extrusion/spheronization technique (pellets) and by direct compression method (minitablets) respectively.

2. To develop and characterize fast-disintegrating enteric-coated pellets and minitablets containing antigen-loaded yeast carriers as vaccine delivery system for oral administration, via extrusion/spheronization technique (pellets) and by direct compression method (minitablets) respectively.
References


www.lcicorp.com (cited 25-4-2010).

CHAPTER 2:

DEVELOPMENT OF A MULTIPARTICULATE FORMULATION CONTAINING STABILIZING AGENTS FOR IMPROVED COLLOIDAL STABILITY IN THE BEER BREWING PROCESS

2.1. Introduction

Colloidal stability of beer can be obtained through the use of polyvinylpolypyrrolidone (PVPP) or tannic acid. The use of stabilisation products during beer filtration is an important procedure in brewing processes that determines the shelf life of beer and hence preserves its stability and quality improvement.

Colloidal instability in beer caused mainly by interactions between haze-forming polyphenols (proanthocyanidins) and haze-sensitive proteins (polypeptides) during the shelf life (long term storage) is a persistent problem facing beer brewers and can result in irreversible non-biological haze and therefore limit the product’s shelf life.

2.2. Main sources of haze precursors

Raw materials and process stages are the two area sources of these haze-forming precursors in packaged beer. Several steps are involved in the brewing process which include, malting, milling, mashing, lautering, boiling, fermenting, conditioning, filtering and packaging.

Polypeptides which are responsible for haze-active proteins are high molecular weight compounds originating mainly from barley and are rich in amino acids proline and glutamic acid, and heavily glycosylated with glucose accounting for 3-7% of total beer protein (Leiper et al., 2003). These polypeptides, known as sensitive proteins, will precipitate with tannic acid, which provides a means to determine their levels in beer.
Proline sites in the haze-active polypeptides bind to the gallotannins so that haze-forming proteins are selectively adsorbed.

Polyphenols in beer originate from barley and hops. Their structure is based on phenol (monohydroxylated benzene) and the term covers all molecules with two or more phenol rings and beer contains approximately 100-300mg/L polyphenol (McMurrough and O’Rourke, 1997). These polyphenols can be divided into derivatives of hydrobenzoic and hydroxycinnamic acids and flavanols and their derivatives. Flavanols and their derivatives account for 10% of total beer polyphenols and contain the species related to colloidal instability (Leiper et al., 2005). Flavanols found in beer are catechin, epicatechin, gallocatechin and epigallocatechin (Figure 2.1), which all exist as monomers but are more commonly joined to form flavonoids as dimers, trimers or larger polymers. Flavonoids found in beer consist of monomers, dimers and a few trimers at a level of approximately 15mg/L. Two dimers that have been associated with haze formation are procyanidin B3 (catechin-catechin) and prodelphinidin B3 (gallocatechin-catechin) (Figure 2.1). These are known as proanthocyanidins and come from malt and hops accounting for 3.3% of total beer polyphenols (McMurrough and O’Rourke, 1997).

![Figure 2.1: Structures of the main flavanol monomers and dimers (Leiper et al., 2005).](image)

Polyphenols that are polymerized and that can precipitate with proteins are called tannins. Both barley and hops contain condensed tannins or proanthocyanidins and these represent
the essential substances responsible for colloidal instability, i.e. they are the haze-active polyphenols (Hannes et al., 2007). Polypeptides and polyphenols combine to produce visible haze that reduces beer’s physical shelf life. The haze proteins have regions rich in the amino acid proline, to which the polyphenols attach. Reducing the levels of one or both of the haze precursors using suitable stabilizing treatments will extend physical stability. The instability can manifest as chill haze (haze formed at a temperature lower than 4 degrees Celsius) or as permanent haze (haze formed at room temperature) (Leiper et al., 2005; Rehmanji et al., 2005; Hannes et al., 2007). Since the two major components of colloidal haze are the protein and polyphenol fractions, their reduction in beer prior to packaging is the obvious target.

2.3. Strategies to minimize haze precursors formation

Colloidal stabilisation of beer by selective removal of either one or both of the haze precursors with suitable stabilizing treatments is the key approach to extend the shelf life. Most widely employed agents in current use for stabilization are PVPP for polyphenol haze precursor reduction and gallotannin for protein haze precursor reduction (Rehmanji et al., 2005). The technique is done by first adding gallotannin powder that adsorbs haze-active proteins by hydrogen bonding followed by addition of PVPP micronized powder that adsorbs tannoids or proanthocyanidins and haze polyphenols through H-bonding between the proton donor (hydroxyl groups) from polyphenols and carbonyl groups from PVPP (Figure 2.2).

![Figure 2.2: Structure of Polyvinylpyrrolidone/Polyvinylpolpyrrolidone (PVPP).](http://en.wikipedia.org/wiki/Crospovidone)

The use of PVPP results in a decrease of the amount of haze-active proanthocyanidins (and monomeric catechins) without a negative influence on beer foam stability (Hannes
The addition of these compounds is done during beer filtration for a specified contact time period.

Other options for achieving good colloidal stability that are being investigated include a novel upstream beer colloidal stabilization employed in the brewhouse toward the end of wort boiling. Upstream beer stabilization technique is easier to employ because the stabilization product is added directly to the boiling wort without the need for specialized equipment, such as slurry tanks and dosing units. This has the advantage of improving wort clarity as well as increasing the shelf life of the beer and thus provides the most effective colloidal stabilization (Rehmanji et al., 2005). Wort is the extracted liquid from the mashing process and contains sugars (mainly maltose & maltotriose from starch in malted barley, and other compounds) that will be fermented by the brewing yeast to produce alcohol.

Recent studies have indicated that the combined use of PVPP and tannic acid (Figure 2.3) at the end of wort boiling has a positive impact on colloidal and flavour stability. The use of tannic acid, one minute later followed by PVPP, ensures good shelf life of the beer. A combined product which ensures that the tannic acid come firstly in contact with the boiling wort and short time later the PVPP, is a straightforward application type for the brewery sector (Hannes et al., 2007).

![Structure of Tannic acid](http://www.chemblink.com/products/1401-55-4)

**Figure 2.3:** Structure of Tannic acid (http://www.chemblink.com/products/1401-55-4)
2.4. Aim of the study

Therefore, the aim of this study was to develop a combined product (PVPP with gallotannin) in form of multiparticulates (pellets or minitablets) that would provide colloidal stabilization as a single product when added before the end of wort boiling and thus introduce the concept of a balanced reduction of both major classes of haze precursors—polyphenols and proteins.

PVPP and gallotannin are incompatible and therefore, a primary coating polymer or layering solution should be sprayed first on the PVPP pellets/minitablets that will separate the two compounds before gallotannin is sprayed in order to develop as single product which ensures that the tannic acid comes first into contact with the boiling wort and short time later the PVPP.

During this study, various PVPP-based pellets with varying concentrations were formulated and tested for disintegration in order to comply with the required contact time of the adsorbent in tank or at the filter point (7 minutes exposure of PVPP in contact with boiling wort) before combining with gallotannin by fluid bed coating to finally develop a combined product. Minitablets (5mm diameter) were also formulated for the same study.

2.5. Materials and Methods

2.5.1. Production of PVPP-based pellets

Pellets were prepared via the extrusion-spheronization technique. Each batch (batch size: 300g) contained polyvinylpolypyrrolidone (Polyclar® 10) (60% - 95%w/w) and additional excipients at varying concentrations. The additional excipients were added in various amounts (between 5% - 40%w/w) so that the prepared pellets disintegrate fast enough (within 7 minutes in boiling simulated wort solution).
The following materials were used during preliminary tests: PVPP (Polyclar® 10; 40 µm and 100µm particle size) supplied by ISP (Switzerland) A.G. Other materials incorporated at various amount included: Unipure EX® starch, potato starch (C*Gel 3002), sodium bicarbonate, glucose monohydrate, sodium starch glycolate (Explotab®), microcrystalline cellulose (Avice© PH 102), sodium alginate, silicon dioxide hydrate, kappa carrageenan, lambda carrageenan, iota carrageenan, microcrystalline cellulose & sodium carboxymethylcellulose (Avicel®RC 581), polyvinyl pyrrolidone (Kollidon 30®), and hydroxypropylmethylcellulose. Demineralised water was used as granulating liquid.

In all prepared formulations, powdered PVPP (Polyclar® 10) with corresponding ingredients (batch size: 300g) were dry mixed for 15 minutes using a planetary mixer (Kenwood Chief, Hampshire, UK) with a K-shaped mixing arm to obtain a uniform powder blend. Specified amount of granulating liquid (demineralised water) was added at once to the powder mixture in the same mixer and blended for 5 minutes to form a wet granulated mass ready for extrusion. The wet mass was extruded axially at a speed of 50 rpm using a single screw extruder (Dome extruder, Lab model DG-L1, Fuji Paudal, Tokyo, Japan) fitted with dome-shaped screen of 1mm diameter perforations and 1.2mm thickness. The formed extrudates were spheronized for 5 minutes in a spheronizer (Caleva Model 15, Caleva, Sturminster Newton, Dorset, UK) with a cross-hatched friction plate at a speed of 850 rpm to form pellets followed by drying in an oven at 40°C for 24hrs.

2.5.2. Production of PVPP minitablets

Minitablets of 5mm diameter containing polyvinylpolypyrrolidone (Polyclar® 10), of 40µm and 100µm particle sizes, and Lambda Carrageenan were also prepared by direct compression of uniform granules.

PVPP and Lambda carrageenan were dry mixed (batch size 100g) for 15 minutes in a Turbula® mixer (model T2A, W.A. Bachofen, Basel, Switzerland) to obtain a uniform
powder mixture. Demineralized water was added to the powder blend and mixed for 5 minutes in a mortar and pestle to form wet granules that were sieved through 1480μm mesh and left to dry for 24 hours at ambient temperature condition.

Dried granules (50g) were sieved through 750μm mesh followed by addition of magnesium stearate. After mixing in a turbula mixer for 15 minutes, the granules were tableted at 25 - 60 kgf (kilogram-force, measured by digital indicator, AD-4532A connected to the tablet press) using a single punch eccentric tablet press (type EKO, Korsch, Berlin, Germany).

**Table 2.1a:** Composition of PVPP-based granules

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyvinylpolypyrrolidone (Polyclar® 10)</td>
<td>80.0</td>
</tr>
<tr>
<td>Lambda Carrageenan</td>
<td>20.0</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>90.0</td>
</tr>
</tbody>
</table>

**Table 2.1b:** Composition of PVPP minitablets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVPP-Lambda Carrageenan dried granules (750 μm)</td>
<td>50.0</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>0.25</td>
</tr>
</tbody>
</table>
2.5.3. Preparation of simulated wort solution

300ml of 12% w/v sucrose solution was prepared and its pH adjusted to 5.0-5.2 by 1% v/v lactic acid and used as a simulated wort boiling solution to determine the disintegration time for the prepared pellets. A pH between 5.0-5.2 is desired in order to obtain clear worts (Withouck et al., 2010).

2.5.4. Pellet disintegration test

100mg of each prepared batch of pellets was dispersed in 300ml of 12% w/v boiling sucrose solution at pH 5.0-5.2 (Figure 2.4a), which was used as a simulated wort boiling solution in this study. Dispersed pellets were observed for complete disintegration of 7 minutes since this is the time allowed for added PVPP powder to adsorb polyphenols in wort boiling. Similar experiment condition was set for PVPP powder (100mg dispersed in 300ml of 12% sucrose boiling solution, pH 5.0-5.2) (Figure 2.4b) to form dispersion that served as a physical control reference for assessing the complete disintegration of pellets.

![Figure 2.4a](image1.png)  ![Figure 2.4b](image2.png)

**Figure 2.4a**: Disintegration test for 85%PVPP +15% Explotab pellets in 12% sucrose solution.

**Figure 2.4b**: PVPP powder in 12% boiling sucrose solution.
2.5.5. Tablet disintegration test

A similar disintegration test was carried out for the prepared minitablets as explained (in section 2.5.4.) above by placing one tablet as a representative of the batch in 300ml of 12% w/v boiling sucrose solution at pH 5.0-5.2 and record the time taken for disintegration. The test was repeated three times.

2.5.6. Tablet hardness test

A sample of minitablets (n=20) (compressed at 20 - 30kgf) was tested for hardness test using a tablet hardness tester, (Type PTB 311, Pharma Test, Hainburg, Germany) and the method as described in European Pharmacopoeia, 6th edition. The test jaws constant speed was 1.00 mm/sec. Results were expressed as the mean, minimum and maximum values of the forces measured, all expressed in Newtons representing twenty determinations.

2.5.7. Tablet friability test

Minitablets (n=20) from the same batch (compressed at 20 - 30kgf) were tested for friability as described in European Pharmacopoeia, 6th edition monograph for friability of uncoated tablets using a friabilator, (Type PTF, Pharma Test, Hainburg, Germany). A sample of twenty minitablets (Ws) was weighed and placed on a sieve 1000 µm size to remove any loose dust with the aid of air pressure before being placed in a friability drum and fitted to a friabilator. The sample was subjected to roll and fall by rotating the drum 100 times and then followed by removal of any loose dust generated from the minitablets during the test. The tablets were weighed again to determine their final weight (Wf). The friability was expressed as the loss of mass and was calculated as a percentage of the initial mass using the following equation:

\[ \text{Friability} \% = \left[ \frac{(W_s - W_f)}{W_s} \right] \times 100 \]  

(Equation 2.1)
2.6. Results and discussion

2.6.1. Pellet disintegration test

PVPP-based pellets that were prepared and tested for disintegration in simulated wort boiling, 12% sucrose soln, pH 5.0-5.2 are presented in Table 2.1. During extrusion process majority of the prepared batches produced extrudates that were plastic and rigid enough to withstand spheronization process and spheronized well to produce spherical pellets, but could not disintegrate completely in 7 minutes which is the recommended contact time. Contact time is the time that the stabilization product is in contact with the wort at the end of boiling (Hannes et al., 2007).

On the other hand, some batches produced plastic and rigid extrudates, but could not form spherical pellets during spheronization and hence ended in forming short rodlike particles. Other materials did not spheronize at all probably lacking strong bonding forces that contribute to pellet formation (Ghebre-Sellassie, 1989b).
Table 2.1: Summary of prepared PVPP-based pellets tested for disintegration test in 300 ml, 12% boiling sucrose solution.

<table>
<thead>
<tr>
<th>Batch no</th>
<th>Preparations name</th>
<th>Extrudates quality</th>
<th>Pellets shape</th>
<th>Observation/results</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>95% PVPP (40µm) + 5% unipure® extrastarch</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>002</td>
<td>90% PVPP (40µm) + 10% MCC</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>003</td>
<td>90% PVPP (40µm) + 10% C*Gel 3002</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>004</td>
<td>90% PVPP (40µm) + 5% unipure® extrastarch + 5% Sodium bicarbonate</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>005</td>
<td>90% PVPP (40µm) + 10% Sodium bicarbonate</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>006</td>
<td>100% PVPP (40µm)</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>007</td>
<td>80% PVPP (40µm) + 20% Glucose monohydrate</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>008</td>
<td>70% PVPP (40µm) + 30% Glucose monohydrate</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>009</td>
<td>85% PVPP (40µm) + 15% Explotab®</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Disintegrated completely</td>
</tr>
<tr>
<td>010</td>
<td>85% PVPP (40µm) + 5% Glucose monohydrate + 5% Explotab® + 5% Sodium bicarbonate</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>011</td>
<td>100% PVPP (40µm) (extruded at 10rpm)</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td></td>
<td>Formulation</td>
<td>Rigidity</td>
<td>Shape</td>
<td>Disintegration</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------</td>
<td>-------------------</td>
<td>----------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>012</td>
<td>80% PVPP(40µm) + 20% Unipure® ex starch</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>013</td>
<td>80% PVPP(40µm) + 20% Sodium Alginate</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>014</td>
<td>80% PVPP(40µm) + 20% Silicon dioxide hydrate</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>015</td>
<td>80% PVPP(40µm) + 20% Kappa Carrageenan</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>016</td>
<td>80% PVPP(40µm) + 20% Lambda Carrageenan</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Disintegrated completely</td>
</tr>
<tr>
<td>017</td>
<td>85% PVPP(40µm) + 15% Lambda Carrageenan</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>018</td>
<td>90% PVPP(40µm) + 10% Lambda Carrageenan</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>019</td>
<td>95% PVPP(40µm) + 5% Lambda Carrageenan</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>020</td>
<td>100% PVPP (100µm)</td>
<td>short soft extrudates</td>
<td>Did not spheronize</td>
<td>Not tested</td>
</tr>
<tr>
<td>021</td>
<td>80% PVPP(100µm) + 20% Lambda carrageenan</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Disintegrated completely</td>
</tr>
<tr>
<td>No.</td>
<td>Formulation</td>
<td>Shape and Disintegration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>-------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>022</td>
<td>75% PVPP (100µm) + 20% Unipure®ex starch + 5% HPMC</td>
<td>rigid enough, spherical, Slightly disintegrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>023</td>
<td>70% PVPP (100µm) + 20% Lambda carrageenan + 10% Unipure®ex starch</td>
<td>rigid enough, short rodlike particles, Disintegrated completely</td>
<td></td>
<td></td>
</tr>
<tr>
<td>024</td>
<td>80% PVPP (100µm) + 10% Kollidon 30® + 10% HPMC</td>
<td>rigid enough, spherical, Slightly disintegrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>027</td>
<td>70% PVPP (40µm) + 20% Lambda carrageenan + 10% Kappa carrageenan</td>
<td>rigid enough, short rodlike particles, Disintegrated completely</td>
<td></td>
<td></td>
</tr>
<tr>
<td>028</td>
<td>80% PVPP (40µm) + 20% Iota carrageenan</td>
<td>rigid enough, short rodlike particles, Slightly disintegrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>029</td>
<td>80% PVPP (40µm) + 20% Lambda carrageenan</td>
<td>rigid enough, short rodlike particles, Disintegrated completely</td>
<td></td>
<td></td>
</tr>
<tr>
<td>030</td>
<td>90% PVPP (100µm) + 10% Kappa carrageenan</td>
<td>rigid enough, spherical, Slightly disintegrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>031</td>
<td>60% PVPP (100µm) + 20% Lambda carrageenan + 20% MCC (Avicel®PH 102)</td>
<td>rigid enough, short rodlike particles, Disintegrated completely</td>
<td></td>
<td></td>
</tr>
<tr>
<td>032</td>
<td>80% PVPP (100µm) + 20% MCC &amp; Sodium carboxymethylcellulose (Avicel®RC 581)</td>
<td>rigid enough, spherical, Disintegrated completely</td>
<td></td>
<td></td>
</tr>
<tr>
<td>033</td>
<td>80% PVPP (40µm) + 20% MCC &amp; Sodium carboxymethylcellulose (Avicel®RC 581)</td>
<td>rigid enough, spherical, Slightly disintegrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>034</td>
<td>100% Polyclar® Brewbrite (Pvpp &amp; Kappa Carrageenan)</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------------------------------------</td>
<td>--------------</td>
<td>-----------</td>
<td>----------------------</td>
</tr>
<tr>
<td>035</td>
<td>100% Polyclar® Plus 730 (Pvpp + amorphous silica gel)</td>
<td>No extrudates formed.</td>
<td>did not spheronize</td>
<td>Not tested</td>
</tr>
<tr>
<td>036</td>
<td>50% PVPP (100µm) + 50% Unipure®ex starch</td>
<td>Too short and soft.</td>
<td>did not spheronize</td>
<td>Not tested</td>
</tr>
<tr>
<td>037</td>
<td>50% PVPP (40µm) + 50% Unipure®ex starch</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>038</td>
<td>70% PVPP (40µm) + 30% Unipure®ex starch</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>039</td>
<td>68% PVPP (40µm) + 30% Unipure®ex starch + 2% Lambda Carrageenan</td>
<td>rigid enough</td>
<td>short rodlike particles</td>
<td>Slightly disintegrated</td>
</tr>
<tr>
<td>040</td>
<td>80% PVPP (40µm) + 10% Kappa Carrageenan + 10% Avicel®RC 581.</td>
<td>rigid enough</td>
<td>spherical</td>
<td>Slightly disintegrated</td>
</tr>
</tbody>
</table>
PVPP-based pellets that were preliminarily tested for disintegration in simulated wort boiling, 12% sucrose soln, pH 5.0-5.2) and disintegrated completely in 7 minutes and hence comply with the contact time required for the PVPP powder to adsorb polyphenols at the end of wort boiling in tank or at the filter point are presented in Table 2.2.

Table 2.2: Disintegration test results for PVPP-based pellets in simulated wort (12% sucrose solution, pH 5.0-5.2).

<table>
<thead>
<tr>
<th>Batch no</th>
<th>Preparations name</th>
<th>Disintegration time</th>
</tr>
</thead>
<tbody>
<tr>
<td>009</td>
<td>85% PVPP (40µm) + 15% Sodium starch glycolate with Sodium Carboxymethyl Starch (Explotab®)</td>
<td>7 minutes</td>
</tr>
<tr>
<td>016</td>
<td>80% PVPP (40µm) + 20% Lambda Carrageenan</td>
<td>7 minutes</td>
</tr>
<tr>
<td>021</td>
<td>80% PVPP (100µm) + 20% Lambda Carrageenan</td>
<td>7 minutes</td>
</tr>
<tr>
<td>023</td>
<td>70% PVPP (100µm) + 20% Lambda Carrageenan + 10% Unipure® ex starch</td>
<td>7 minutes</td>
</tr>
<tr>
<td>027</td>
<td>70% PVPP (40µm) + 20% Lambda Carrageenan + 10% Kappa Carrageenan</td>
<td>7 minutes</td>
</tr>
<tr>
<td>031</td>
<td>60% PVPP (100µm) + 20% Lambda Carrageenan + 20% Microcrystalline cellulose (Avicel® PH 102)</td>
<td>7 minutes</td>
</tr>
<tr>
<td>032</td>
<td>80% PVPP (100µm) + 20% Microcrystalline cellulose &amp; Sodium carboxymethylcellulose (Avicel®RC 581)</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>

Preparations, batch no 009 and 032 were tested in real wort (malted barley and hops) laboratory scale trials and their results are presented in Table 2.3. The results are presented in percentage concentration using blank (100% wort without PVPP treatment)
as a standard reference. The tested real wort was treated with PVPP-based pellets at 10 g/hl for contact time of 7 minutes.

During wort treatment with PVPP-based products (powder, pellets or tablets), attention is paid to the reduction of proanthocyanidins content, the less proanthocyanidins, the better. The polyphenol concentration gives an overview of the total polyphenols (partly proanthocyanidins and flavonoids) present in the wort. The sensitive proteins are removed by addition of gallotannin as mentioned earlier. PVPP is considered to be effective when proanthocyanidins level is reduced between 30 - 40% with reference to the blank.

**Table 2.3:** Effectiveness of PVPP-based pellets in reducing proanthocyanidins concentration in real wort laboratory scale trials

<table>
<thead>
<tr>
<th>Batch</th>
<th>Flavonoids</th>
<th>Polyphenols</th>
<th>Proanthocyanidins</th>
<th>Sensitive proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>009</td>
<td>95%</td>
<td>95%</td>
<td>88%</td>
<td>126%</td>
</tr>
<tr>
<td>032</td>
<td>97%</td>
<td>98%</td>
<td>96%</td>
<td>106%</td>
</tr>
</tbody>
</table>

According to the results obtained from the wort laboratory scale test shown in Table 2.3, there was a reduction in the level of proanthocyanidins (88% and 96%) when the wort was treated with 85% PVPP + 15% Sodium starch glycolate (Explotab®) and 80% PVPP + 20% Microcrystalline cellulose & Sodium carboxymethylcellulose (Avicel®RC 581) pellets, respectively in relation to blank. However, the reduction was not sufficient enough and therefore, it was decided to develop a combination of minitablets containing PVPP and lambda-carrageenan. PVPP + lambda-carrageenan was the only combination that disintegrated completely in 7 min, but it was not possible to make pellets of this combination as they formed short rodlike particles, and hence not spheronsable (Table 2.1, batch no. 021).
It is worthwhile to mention that carrageenan, which is a natural product extracted from red marine algae, has a long history of use in brewing as a wort clarifier. It is effective in reducing haze-precursors in wort and these include protein, polyphenol, polysaccharide and other materials (ISP: Polyclar® BrewbriteTM, 2004, version Jan-05). Therefore, its inclusion in these preparations also reduces the formation of protein-polyphenol complexes.

2.6.2. Tablet disintegration test

Minitables (80% PVPP (40µm) + 20% Lambda Carrageenan, and 80% PVPP (100µm) + 20% Lambda Carrageenan) disintegrated in 7 minutes when preliminarily tested in simulated wort (12% sucrose solution) as described earlier and hence achieved the disintegration test to comply with the time required when PVPP is dispersed in wort at the end of boiling.

The prepared minitablets were further tested in real wort produced at laboratory scale and the results are presented in Table 2.4.

Table 2.4: Effectiveness of PVPP-based minitablets in reducing proanthocyanidins concentration in real wort laboratory scale trials

<table>
<thead>
<tr>
<th></th>
<th>Flavonoids</th>
<th>Polyphenols</th>
<th>Proanthocyanidins</th>
<th>Sensitive proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMT1</td>
<td>101%</td>
<td>99%</td>
<td>68%</td>
<td>128%</td>
</tr>
</tbody>
</table>

The tested real wort was treated with PVPP-based tablets at 15g/hl for contact time of 7 minutes.

Proanthocyanidins level was reduced to 68% (a reduction of 32% in relation to blank, which is 100% wort without PVPP addition as reference standard) when minitablets
containing 80% PVPP and 20% Lambda Carrageenan were used. This indicated that PVPP was effective.

2.6.3. Tablet hardness test

The hardness of a tablet refers to a measure of the crushing/breaking force under diametrical loading. The mean, minimum and maximum measured forces for the tested minitablets \((n=20\); compressed at 20-30kgf) were 23N, 18N and 33N respectively. Since the hardness of a tablet affects its disintegration time, the harder the tablet, the longer it takes to disintegrate. The obtained values are considered acceptable in relation to this study since the minitablets disintegrated in 7 minutes as required.

2.6.4. Tablet friability test

Friability refers to the tendency for the tablets to form dusts or to break-up/off when subjected to abrasion forces. It is the ability of the compressed tablet to avoid fracture and breaking apart during transport.

A prepared combination of 80% PVPP and 20% Lambda Carrageenan minitablets (compression force, 20-30kgf) had a high mechanical strength, since friability values of less than 1% were obtained \((\text{friability} = 0.73\%)\). The pharmacopeial limits consider a maximum loss of 1 percent of the mass of the tablets tested to be acceptable. This low friability indicates the tablet’s ability to withstand the shear forces when subjected to mechanical shock or attrition.
2.7. Conclusion

Preliminary results indicate that the composition of the minitablets containing 80% PVPP and 20% lambda-carrageenan has managed to reduce the formation of polyphenols in worts on laboratory scale. These results will pave the way to the next step of developing a combined product that will be applied in brewing trials and implemented in several industrial “case” studies in order to achieve colloidal stability by reducing these protein-polyphenol complexes. By using fluid bed coating technique, the PVPP minitablets will first be coated with maltodextrin solution and finally, coated with gallotannin.

Prepared PVPP-based pellets, though tested, could not reduce significantly the amount of haze-forming precursors after the required disintegration time in wort boiling.


http://www.thoroughbrews.com/pb/wp_b120e579/wp_b120e579.html (cited on 6-5-2010)


CHAPTER 3:

FORMULATION OF ENTERIC COATED PELLETS AND MINITABLETS CONTAINING ANTIGEN-LOADED YEAST CARRIERS

3.1: Vaccination

Vaccination was introduced by Jenner (1749 –1823) and Pasteur (1822 – 1895) in the late 18th and 19th centuries using attenuated or killed microorganisms to induce protective immunity against several pathogens (Geison, 1978; Stewart and Devlin, 2006). The methods are still applicable to date and have led to the control of infectious diseases such as smallpox, diphtheria, tetanus, pertussis, polio and rabies worldwide (WHO/IVB/2009).

A vaccine is a biological preparation typically containing an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe or its toxins to be administered to humans or animals in order to improve immunity to a particular disease. All vaccines work by presenting a foreign antigen to the immune system in order to evoke an immune response. The agent stimulates the body’s immune system to recognize the agent as foreign, destroy it, and remember it, so that the immune system can more easily recognize and destroy any of these microorganisms that it will later encounter. The pathogen is made harmless without loss of antigenicity and therefore allows an individual to acquire specific immunity to an infectious agent without having to suffer an initial infection. When administered, it induces antibodies against the pathogens or toxins and also induces a cellular response and thus generates a strong immune response to provide long term protection against infection.

Despite the efficacy of killed and attenuated vaccines, there is still a concern over their safety. Killed bacterial or viral vaccines often have residual toxicity following inactivation and might contain toxic components, such as lipopolysaccharide (LPS).
Attenuated live vaccines may induce disease via reversion or mutation to an infectious agent to people with an impaired immune system (Perrie, 2006). This has changed the focus on vaccine development to safer, although often less immunogenic, subunit vaccines composed of purified antigenic components of the microorganism. Attenuated live vaccines generally possess a natural adjuvant capability that is built on the ability of our immune system to recognize many potential dangerous microbes and thus contribute to the control of infectious diseases to the immunized population (Aguilar and Rodriguez, 2007). Unlike attenuated live vaccines, highly purified recombinant molecules or subunits of pathogens lacks inherent immunostimulatory property of the pathogens, and thus do not often elicit strong immune responses to reach their full potential.

Consequently, the development of subunit vaccines composed of purified antigenic components or recombinant molecules of the microorganism would require the addition of an adjuvant in order to be effective (Petrovsky and Aguilar, 2004). The term “adjuvant” is derived from the Latin word adjuvare which means to help. Any material that helps the antigens or increases the humoral and/or cellular immune response to an antigen is referred to as an immunologic adjuvant. Adjuvants have been in use to augment the immune response to antigens (Gupta and Siber, 1995).

The only universally licensed adjuvant for human vaccine is an Aluminium-based mineral salt (alum) (Gupta and Siber, 1995; Aguilar and Rodriguez, 2007) which is widely used in Diphteria, Tetanus, Pertussis and Hepatitis A and B vaccines (Vogel et al., 1995). Alum’s success as adjuvant is associated with enhanced antibody responses and its mechanism has recently been revealed to act through activation of the Nacht Domain-, Leucine-Rich Repeat (LRR) -, and PYD-Containing Protein 3, (NALP3) inflammasome by induction of uric acid (Kool, 2008). Although alum enhances antibody responses to parenterally delivered vaccines, it is not effective at inducing cellular immunity and is not suitable for mucosal immunization (Moore et al., 1995). It is also faced with cumulative aluminium toxicity and induction of allergic immunoglobulin-E (IgE) antibody responses (Aggerbeck et al., 1995; Gupta, 1998).
Ideally, adjuvants are required to be strong enough to stimulate B- and T-cell immunity while avoiding the excess activation of innate immune system and inflammatory cytokine production that mediates adjuvant reactions and side effects to a patient (Petrovsky, 2008). Adjuvant side effects can be grouped into local and systemic reactions. Local side effects range from increased injection site pain, inflammation and swelling, granulomas, sterile abscess formation, lymphadenopathy and ulceration. Systemic vaccine reactions may include nausea, fever, adjuvant arthritis, uveitis, eosinophilia, allergic reactions, organ-specific toxicity, anaphylaxis or immunotoxicity mediated by liberation of cytokines, immunosuppression or induction of autoimmune diseases (Waters et al. 1986; Aguilar and Rodriguez, 2007).

A new vaccine adjuvant can be approved by the regulatory body if it clearly demonstrates its safety, tolerability and acceptability that outweigh any increased reactions. Hence, a major challenge in adjuvant development is how to achieve a potent adjuvant effect while avoiding reactions or toxicity (Gupta and Siber, 1995). Thus, the need to develop novel adjuvants against pathogens that have not been managed (e.g. Malaria and HIV/AIDS) by the traditional vaccination strategies and to overcome the limitations of the available licensed adjuvants for human vaccines is vital (Gupta, 1998; Harandi et al., 2010).

Despite that, aluminum salt-based adjuvants continue to be the only adjuvants licensed for human vaccines delivery system, natural and synthetic compounds have also been identified that have adjuvant activity. The goal at the development of new vaccine delivery systems is to identify optimal methods for presenting target antigens to the immune system that will elicit immune responses appropriate for protection against, or treatment of, a specific disease. A number of novel adjuvants, which may be used to supplement or replace alum in human vaccines, have been under development and in preclinical evaluation for several decades (Gupta and Siber, 1995; Ryan et al., 2001; Moingeon et al., 2002; Aguilar & Rodriguez, 2007). Despite the enormous number of candidates tested, alum has not been replaced in human vaccines because the potency of these candidates has been regularly associated with increased toxicity, and this has barred their use, particularly in prophylactic vaccines where safety issues are vital. Therefore,
there is still a major need for the development of safe and efficacious adjuvant that is capable of boosting cellular and humoral immunity (Gupta, 1998; Petrovsky, 2006).

Hence, the aim of this study was to develop enteric coated pellets and minitablets formulation containing antigen-loaded yeast carriers/shells as delivery system for oral vaccination via extrusion/spheronization and direct compression methods respectively. The yeast shells to be used as carriers of antigen were extracted from Baker’s yeast (Saccharomyces cerevisiae) cell walls that contain yeast-beta glucan as the main component.

3.2: Structure and classification of glucans.

Glucans are natural polysaccharides comprised of glucose monomers linked by glycosidic bonds (Figure 3.1). They are classified according to the type of intra-chain linkage of the polymer into alpha (α-) and beta (β) - glycosidic bonds, depending on whether the substituent groups on the carbons flanking the ring oxygen are pointing in the same or opposite directions.

![Figure 3.1: General structure of glucan (Novak and Vetvicka, 2008).](image)

An alpha glucan (α-glucan) has α-glycosidic bond which emanates below the plane of the sugar, whereas the hydroxyl (or other substituent group) on the other carbon points above the plane (opposite configuration) (Figure 3.2a). The beta glucan (β-glucan) has a β-glycosidic bond which emanates above that plane (Figure 3.2b).
D-glucan (highly branched polymer of glucose, Figure 3.3b).

Among the common alpha-D-glucans include, α-(1,6)-D-glucan (Dextran); α-(1,4)-D-glucan (Amylose-mostly linear polymer; Figure 3.3a) and Amylopectin which is α-(1,4)-D-glucan (highly branched polymer of glucose, Figure 3.3b).

Naturally, β-Glucans occur as glucose polymers, consisting of a backbone of β-(1,3)-linked by β-D-glucopyranosyl units with β-(1,6)-linked side chains of varying distribution and length (Figure 3.5). These polysaccharides are commonly named “β-glucans” although they are chemically heterogeneous, and the one referred in this work, are non-cellulosic polymers of β-D-glucose, with glycosidic bonds in position β-(1,3).
3.3 Sources of glucans

Glucans are found naturally as highly conserved cell wall structural components in mushrooms (i.e. Maitake, Reishi, and Shiitake –Figure 3.4), yeast, bacteria, seaweed/algae and wheat (i.e. cereal grains of oats and barley). The beta-linked glucans, predominantly, β-(1,3)-D-glucans are found in fungi (Wasser, 2002; Brown and Gordon, 2003).

![Figure 3.4: Edible mushrooms, e.g. shiitake (Lentinula edodes), a source of natural beta-glucans.](http://en.wikipedia.org/wiki/Beta_glucan)

The cell wall of the common baker’s or brewer’s yeast, Saccharomyces cerevisiae (S. cerevisiae) is one of the readily available sources of β-(1,3)-D-glucan. S. cerevisiae, being yeasts (unicellular fungi), are commonly used in bakery and beer industries for baking and beer fermentation respectively for many years (Otero et al., 1996). Other fungal and bacterial sources of β-glucans with immunomodulatory effects are listed in Table 3.1.
Table 3.1: Common used biologically active β-glucans from fungal and bacterial sources (Novak and Vetvicka, 2008).

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curdlan</td>
<td>Bacteria; Alcaligenes faecalis</td>
</tr>
<tr>
<td>Laminaran</td>
<td>Seaweed; Laminaria sp</td>
</tr>
<tr>
<td>Pachymaran</td>
<td>Poria cocos</td>
</tr>
<tr>
<td>Lentinan</td>
<td>Lentinus edodes</td>
</tr>
<tr>
<td>Pleuran (HA-glucan)</td>
<td>Pleurotus ostreatus</td>
</tr>
<tr>
<td>Schizophyllan</td>
<td>Schizophyllum commune</td>
</tr>
<tr>
<td>Selerotinin (SSG)</td>
<td>Selerotinia sclerotiorum</td>
</tr>
<tr>
<td>Scleroglucan</td>
<td>Sclerotium glucanicum, S. rolfsii</td>
</tr>
<tr>
<td>Grifolan</td>
<td>Grifola frondosa</td>
</tr>
<tr>
<td>Yeast glucan</td>
<td>Saccharomyces cerevisiae</td>
</tr>
</tbody>
</table>

3.4 Composition of fungal cell walls

The fungal cell walls are composed mostly of β-(1,3)-glucans and mannoproteins with branched β-(1,6)-glucan that links the components of the inner and outer walls. Other minor component includes chitin, which contributes to the insolubility of the fibers. The β-(1,3)-glucan-chitin complex is the major constituent of the inner wall while the outer
surface of the wall are mannoproteins. Other components found and covalently linked to a great extent include glycoprotein, lipids, and carbohydrates such as mannan (Lipke and Ovalle, 1998; Klis et al., 2002; Chan et al., 2009) (Figure 3.5).

![Diagram of fungal cell wall components and β-D-glucan structure](image)

**Figure 3.5:** Components of the fungal cell wall and the structure of β-D-glucan showing a linear backbone of β-(1,3)-linked glucose with β-(1,6)-linked side branches (Chan et al., 2009).

The first investigation and preparation of yeast-glucan from the cell wall of baker’s yeast was reported by Pillemer and Ecker (1941), who revealed the presence of a glucan-enriched product called Zymosan in the cell wall. Other studies that supported the presence of zymosan in *S. cerevisiae* were done by Bacon and Farmer, (1968) and Manners et al., (1973). They found that, the major component (about 85 %) is a branched β-(1,3)-glucan of high molecular weight (about 240,000 or 240kDa) containing 3% of β-
(1,6)-glucosidic interchain linkages and the minor component is a branched β-(1,6)-glucan. Earlier investigations done by Bacon and Farmer, (1968), reported the presence of a predominantly β-(1,6)-glucan and several types of β-glucans in the yeast cell wall before a detailed fractionation of the cell wall components and their characterization carried by Fleet and Manners, (1976) confirmed the major component of yeast β-glucan to be a backbone chain of high-molecular β-(1,3)-D-glucan and the minor component to be β-(1,6)-linked glucose residues.

3.5 Properties of β-glucans

Glucans are characterized mainly by homopolymers of glucose, the chain length, the kind of glycosidic linkage and the degree of branching. They consist of linear (1,3)-β-linked backbones with either (1,6) or (1,4)-linked side chains of varying length and interval/distribution, which form complex tertiary structures stabilized by interchain hydrogen bonds. The primary structure, solubility, degree of branching (DB), molecular weight (MW), and helical conformation (triple helix, single helix or random coil conformation) are characterizing parameters of β-(1,3)-D-glucan that have been shown to play a role in glucans biological activity (Bohan and BeMiller, 1995; Sandula et al., 1999; Borchers, et al. 1999).

The solubility of the glucan in water depends on the frequency and length of the side chains (Laroche and Michaud, 2007). For example, Yeast glucan from Saccharomyces cerevisiae is insoluble in alkali and water as it is made up of single β-D-glucopyranosyl units on every eight main chain units (Bacon et al., 1969), which cause the glucan to be insoluble in alkali and hence named particulate (“insoluble”) β-glucans.

The frequency and side chain units has also been observed to influence the solubility of schizophyllan, (from Schizophyllum commune fungus) which has a 1,6-D-glucose side chain on every third glucose unit along the main chain (Figure 3.6a), making it completely water soluble (Bae et al. 2004, Okobira et al., 2008). On the other hand,
Figure 3.6: (a) Repeating unit of schizophyllan. (b) Representative model of the triple helix with the balls representing the side chains (Bae et al., 2004).

Curdlan, a linear β-(1,3)-D-glucan from bacteria, Alcaligenes faecalis, which has no side chains, forms water-insoluble triple helical structure in aqueous solution. Depending on the heating temperature, time of heat-treatment and concentration, curdlan has the ability to form gels with various strength and thus used as thickening and gelling agent in foods (Harada et al., 1968; Williams, 1991; McIntosh et al., 2005; Laroche and Michaud, 2007).

In order to improve solubility of particulate (“insoluble”) β-glucans, chemically modified particulate β-glucans, i.e. carboxymethylated, sulfonated and phosphorylated β-glucans have also been developed. This has led to the formulation of derivatized particulate β-glucans that can be administered parenterally compared to particulate (“insoluble”) β-glucans which causes adverse effects like inflammation, granuloma formation, emboli formation and pain when administered by parenteral routes (Sandula et al., 1999; Zeković et al., 2005; Kanlayavattanakul.M. and Lourith.N, 2008).

The differences between β-glucan linkages and chemical structure are also important as far as solubility, mode of action, and overall biological activity are concerned (Bohn and
The branching assignments also appear to be species specific, for example, β-glucans of fungus have (1,6)-side branches whereas those of bacteria have (1,4)-side branches (McIntosh et al., 2005).

The helical conformation of β-(1,3)-glucans, which includes triple helix, single helix, and random coil conformers, is recognized as an important contributing factor in biological activity. β-(1,3)-glucan chains conform to a shape comparable to a flexible wire spring (Figure 3.6b), that can exist in various states of extension (Klis et al., 2002). Various studies conducted have confirmed that β-glucans assume conformational change into triple helix, single helix or random coils in aqueous solution (Takeda et al., 1978; Williams, 1991; Bohn and BeMiller, 1995; Sandula et al., 1999; Bae et al., 2004; Zhang et al., 2005; Surenjav et al., 2006; Okobira et al., 2008). In addition Thammakiti et al., 2004 found that, β-glucans have high apparent viscosity, water holding, oil binding, and emulsion stabilizing capacities. The triple helix conformation (figure 2.6b) is stabilized by hydrogen bonding at the C-2 hydroxyl, (Deslandes and Marchessault, 1980; Tadashi et al., 2008). Researchers have demonstrated that, the immune functions of β-glucans depend on their stable helix conformational complexity (Zhang et al., 2007).

### 3.6 Immune-enhancing properties of glucans

The role of Yeast-derived β-(1,3)-glucans as biologically active immunomodulator has been widely documented. Interest in the immunomodulatory properties of these polysaccharides was initially raised after investigations done by Pillemer and Ecker, (1941) using baker’s yeast, Saccharomyces cerevisiae to demonstrate that, a crude yeast cell wall extract (Zymosan) was responsible for stimulation of macrophages via activation of the complement system.
Yeast-derived β-(1,3)-glucan is classified as ‘Biological Response Modifier’ (BRM), which means, it modifies the host’s biological response by stimulating the immune system (Bohn and BeMiller, 1995; Sandula et al., 1995; Rice et al., 2002; Underhill, D.M., 2003). The β-glucans stimulate the host’s defense mechanisms against disease challenge rather than attacking the infective or tumor-causing agent, and therefore remains nontoxic to the cells of the host organism. Biological response modifiers can be grouped into two categories according to their effects: cytokines, which are responsible for communication between immune system cells and regulation of the system, and immunomodulators, which are responsible for immunopotentiation (i.e. positive maneuver) or immunosuppression (i.e. negative maneuver) of the immune system (Novak and Vetvicka, 2008).

The exact mechanism of action of glucans remain to date undecided in spite of long history of research as biological modulators and search for optimal chemical configuration is still going on (Freimund et al., 2003; Brown and Gordon, 2003; Zhang et al., 2007; Vetvicka et al., 2008; Novak and Vetvicka, 2009). As β-glucans are natural products in origin, they present a complex mixture of ingredients, each one of which may contribute to their biological activity and thus render challenges in characterization (Novak and Vetvicka, 2008). However, many researchers concur with the conclusion drawn on the structural requirements for a physiological effect of β-(1,3)-D-glucans that, the immunopotentiating activity depends on specific molecular structure such as primary structure, molecular weight, branching patterns, solubility and helix conformational features. Immunopotentiation effected by binding of β-(1,3)-glucan molecule or particle includes activation of cytotoxic macrophages, helper T cells, and Natural Killer (NK) cells, promotion of T cell differentiation, and activation of the alternative complement pathway (Bohn and BeMiller, 1995; Cleary et al., 1999; Ooi VE, and Liu, 2000; Freimund et al., 2003; Zhang et al., 2005).
3.6.1 Interaction of β-glucans with the gastro-intestinal tract

Absorption of orally taken yeast-derived β-(1,3)-glucans and similar compounds has been reported to be facilitated by enterocytes across the intestinal cell wall into the lymph where they interact with macrophages to activate immune function (Frey et al., 1999). M (Microfold) cells within the Peyer’s Patches (Figure 3.7) transport the insoluble whole glucan particles into the Gut-Associated-Lymphoid Tissue (GALT) (Hong et al., 2004).

![Diagram of intestinal lumen showing M (Microfold) cells with the Peyer’s Patches](Volman et al., 2008)

**Figure: 3.7:** section of intestinal lumen showing M (Microfold) cells with the Peyer’s Patches (Volman et al., 2008)

3.6.2 Immunopharmacological activities of β-glucans

Numerous reports have documented the ability of β-(1-3)-glucans to nonspecifically activate cellular and humoral components of the host immune system. Yeast-derived β-(1,3)-glucans and β-glucans from mushrooms work in part by stimulating innate antifungal immune mechanisms to increase host’s resistance to fight a range of pathogenic challenges from bacteria, fungi, parasites, viruses and cancer (Lindequist et al. 2005). Radioprotective and adjuvant effects of yeast-derived β-(1,3)-glucans have also been reported. The mammalian innate immune system can recognize β-glucans as pathogen-associated molecular patterns (PAMPs) since they are not found in mammals. PAMPs
represent small molecular sequences of microbial products, usually found in the cell wall of microorganisms like carbohydrates, lipids, and proteins that are not produced by the host and are recognized by professional Antigen Presenting Cells (APC) like dendritic cells (DC) and macrophages by Toll-like receptors (TLR) and other pattern recognition receptors (PPR) (Kougias et al. 2001; Rice et al., 2002; Brown and Gordon, 2003).

3.6.2.1. Antitumor activity

The ability of glucans to inhibit tumour growth in a variety of experimental tumour models is well established. A wide variety of mushroom species have been investigated for their antitumor activities since Japanese researchers began to investigate systematically whether mushrooms, both wild and cultured, contained substances able to inhibit tumor growth in the late 1960s. Studies in the host animal suggest that the tumor inhibiting activity is contained in the polysaccharide fraction and the activities are related with molecular weight, degree of branching and higher (tertiary) structure of β-glucans. The triple helical conformation plays an important role in enhancement of the antitumor activities (Bohn and BeMiller, 1995; Borchers et al., 1999; Sandula et al., 1999; Zekovi´c et al., 2005; Surenjav et al., 2006).

Some of the isolated compounds with most effective anti-tumor activity include Lentinan from Lentinus edodes, Schizophyllan from Schizophyllum commune, Grifolan from Grifola frondosa, and SSG from Sclerotinia sclerotiorum (Suzuki et al. 1987; Ohno et al. 1987; Ooi VE, and Liu F, 2000; Zhang et al., 2005; Zhang et al., 2007). Yeast β-(1,3)-glucan derived from Saccharomyces cerevisiae (Baker’s yeast) and administered orally in mice has also demonstrated to inhibit the growth of cancer cells in vivo (Vetvicka et al., 2002; Hong et al., 2004).

3.6.2.2 Macrophage–activating ability

Several in vitro and in-vivo studies have shown that β-glucans act on several immune receptors including Dectin-1, complement receptor (CR3) and TLR-2/6 and trigger a
group of immune cells including macrophages, neutrophils, monocytes, natural killer (NK) cells, and dendritic cells (Rice et al., 2002; Brown et al., 2003; Brown and Gordon, 2003; Taylor et al., 2002; Taylor et al., 2007; Novak and Vetvicka, 2008; Chan et al., 2009). They also activate T cells and their cytokine production and hence, both innate and adaptive response are modulated by β-glucans and phagocytosis enhanced (Vetvicka et al., 1996; Borchers et al., 2008; Volman et al., 2008). Following oral administration in animal studies, β-glucans enter the proximal small intestine and some are captured by the macrophages and then internalized and fragmented into smaller sized β-glucan fragments within the macrophages. They are transported by the macrophages to the marrow and endothelial reticular system. The small β-glucans fragments are eventually released by the macrophages and taken up by the circulating granulocytes, monocytes or macrophages via the complement receptor (CR)-3 leading to various immune response (Underhill, 2003; Hong et al., 2004) (Figure 3.8).

Figure 3.8: The uptake and subsequent actions of β-glucan on immune cells (Chan et al, 2009).

3.6.2.3 Radioprotective activity.

The radioprotective (bone marrow protective effect) activities of yeast-derived β-glucans has been well-established and reported in literature and its uptake is via M cells in the intestinal Peyer’s patches (Figure 3.7). The oral uptake of yeast-derived β-glucan particles lead to β-glucan presentation to macrophages in the underlying GALT which is
then transported into the organs of the reticuloendothelial system –RES (lymph nodes, spleen and bone marrow) and thus enhancing resistance to microbial invasion, accelerating bone marrow recovery, restoration of cell formation (hemopoietic recovery) and increased survival after exposure to radiations following injury as demonstrated in irradiated mice studies due to prolonged macrophage function (Patchen and MacVittie, 1983; Patchen and MacVittie, 1986; Patchen et al. 1987; Patchen et al. 1998; Wakshull et al. 1999; Allendorf et al., 2003; Hong et al., 2004; Gu et al. 2005; Cramer et al. 2006; El-Batal et al., 2008).

3.6.2.4 Cholesterol & glycaemia lowering effects

Research also reports that yeast-derived \( \beta \)-glucan fiber are helpful in lowering elevated levels of Low Density Lipoprotein (LDL) cholesterol concentrations and in reducing elevated blood sugar levels. The orally taken beta-glucan has a natural nutritional fiber property and passes the stomach unchanged. They are not degraded due to lack of specific human enzyme (\( \beta \)-1,3-glucanase) that would break it to glucose or di-glucose so that can be absorbed through the intestinal wall. The presence of an unabsorbed high-fiber diet in the intestine increase intestinal viscosity and decrease cholesterol absorption, thereby, promoting cholesterol catabolism and excretion of bile acids and thus imparts a hypocholesterolemic effect. \( \beta \)-glucan as a soluble fiber, delays the digestion of dietary carbohydrates that can have a possible sugar control benefit for a diabetic patient due to this delay (Felix et al., 1995; Brennan et al., 1996; Nicolosi et al., 1999; Frank et al., 2004).

3.6.2.5 Protection against infections

In addition to enhancing the activity of the immune system, \( \beta \)-(1,3)-glucan is also reported to help prevent infections and aid in wound healing. The anti-inflammatory activity of \( \beta \)-(1-3)-glucan has been tested in both animal models and in human for bacterial, fungal, (Meira et al., 1996) protozoal (Tuwajri et al. 1987) and viral (Ara et al., 2001) infection. \( \beta \)-(1-3)-glucans have been evaluated in experimental models of
Escherichia coli, Staphylococcus aureus, Streptococcus pneumonia, (Hetland et al., 2000); Pseudomonas aeruginosa, (Koike, 1976); Mycobacterium leprae (Rayyan and Delville, 1983) and Mycobacterium bovis (Hetland et al, 1998) infection. In each case, treatment with β-(1,3)-glucan had a beneficial protective effect in reducing mortality or decreasing bacterial counts in infected or susceptible animals.

3.6.2.6. β-(1,3)-glucan as an Adjuvant for vaccine

Oral and intradermal administered yeast β-(1,3)-glucan has been demonstrated to serve as a vaccine adjuvant with both conducted experiments suggesting that protein antigens can be conjugated to yeast β-(1,3)-glucan to provide an adjuvant effect for stimulating the antibody response to protein antigens (Berner et al., 2008).

Other studies which have provided evidence for the beneficial adjuvant effects of yeast β-(1,3)-glucan are explained below. Vetvicka et al., (2002) demonstrated that, orally-administered yeast β-(1,3)-glucan had significant effects as a prophylactic treatment to reduce the mortality of anthrax infection in mice when infected subcutaneously with a lethal dose of anthrax spores. In addition, the same type of treatment also inhibited the growth of cancer cells in vivo. The mechanism of action involved the stimulation of three important cytokines: Interleukin-2, (IL-2), Interferon-γ (gamma), (IFN-γ), and Tumour Necrosis Factor-α (alpha), (TNF-α). Ara et al., (2001) conducted a study on the adjuvant effect of zymosan on human immunodeficiency virus type-1 (HIV-1)-specific DNA vaccine by intramuscular coinjection to BALB/c mice with candidate DNA vaccine for HIV-1 and suggested to be an effective immunological adjuvant in DNA vaccination against HIV-1. They suggested that zymosan-mediated DNA vaccination enhanced helper T cell (Th) 1-mediated immunity and the effect was based on its recruitment and activation of macrophages, dendritic cells or antigen-presenting cells (APC) through complement activation. After being activated by zymosan, macrophages induced production of cytokines such as interferon-γ (IFN-γ), interleukin (IL)-2, IL-8, and tumour necrosis factor-α (TNF-α).
The ability of β-glucan to exhibit significant adjuvant activity has also been studied and demonstrated by Williams et al. (1989) when they investigated the activity in immunization against Trypanosoma cruzi, (causative agent of Chagas' disease, that infects humans and animals in tropics). Mice subcutaneously immunized with particulate glucan combined with a vaccine of glutaraldehyde-killed T. cruzi reported to show an increased survival versus controls following days of post-challenge.

β-glucan has also been shown to exert a strong adjuvant effect in experimental immunization when injected simultaneously with Formalin-killed erythrocytic stages of the rodent malaria parasite Plasmodium berghei. Antigen-glucan immunization demonstrated degree of immune protection to the host against the challenge with live parasite (Holbrook et al., 1981; Maheshwari and Siddiqui, 1989). Also an adjuvant effect in experimental immunization trials has been observed in visceral/cutaneous leishmaniasis, disease caused by protozoan parasites Leishmania that infect mammalian macrophages, and cause visceral infection of the reticulo-endothelial system (Tuwaijri et al., 1987). Oral glucan administration has also been demonstrated to increase survival in mice challenged with Staphylococcus aureus or Candida albicans (Rice et al., 2005). In summary, there is research evidence to substantiate β-glucan’s potential as an adjuvant for vaccine delivery.

3.7 Oral delivery of vaccines and delivery systems

Prevention of infections by vaccination has proven to be an efficient and cost-effective strategy for public-health improvement (WHO/IVB/2009). The majority of the available vaccines are administered by intramuscular or subcutaneous injection, which makes mass immunization costly and less safe, particularly in developing countries as it poses safety risks like needle-stick injuries and the transmission of blood-borne diseases. Hence administration of vaccines via oral route will overcome these problems and have large implications since the access to trained medical staff to administer vaccines by injection is limited. Administration of vaccine by oral route could also stimulate immune response
at mucosal surfaces which is not observed in parenteral vaccination (Lavelle, 2005; Simerska et al., 2009).

Induction of protective immunity at the mucosal sites will prevent colonization and translocation of the pathogens across the mucosal barrier since most infectious microorganisms get entry into the systemic circulation through mucosal surfaces. Therefore, oral delivery of vaccines for mucosal immunisation would provide a first line of defence and the potential to prevent systemic infections and at the same time generating secretory IgA (sIgA), which play a major role in mucosal defense responses at all mucosal sites apart from the administration site. Hence oral vaccine has the potential to induce both systemic and mucosal immunity (Challacombe et al. 1992; Shalaby, 1995).

Other advantages that oral vaccines offers compared to parenteral ones are as follows.

Oral administration of vaccines is painless, and therefore pains associated with injections like site reactions are avoided. It is less invasive and less expensive than injectable ones and easy to administer outside formal clinical settings without the need for skilled personnel. It involves only swallowing and hence no possible transmission of infections from contaminated needles and syringes contrary to injectable ones where the risk of infectious disease transmission is possible like blood-borne viral diseases due to use of contaminated needles, reuse and disposal. The ease of oral administration improves vaccine coverage in remote areas and enhances compliance, particularly in children and when multiple doses of vaccines are required (Chen and Langer, 1998). Oral vaccines may be supplemented with adjuvants (Jain et al., 1996) and also may be cheaper to produce as a result of less tough regulatory requirements for preparations compared to injectable ones (Mutwiri et al., 2005). Therefore, oral vaccines are seen as effective prophylactic tool because of better compliance and ability to generate both mucosal and systemic immunity, whereas injectable vaccines generate only systemic immune response (Aziz et al., 2007).
Despite all these potential advantages, less focus has been paid on the development of oral vaccines since the route has been regarded historically to be less effective, as vaccine antigens undergo digestion in the GI tract before inducing an immune response. Another concern is that, the mucosal tissues of the gut is continuously exposed to large amounts of normal flora, microbial and food antigens, and in that respect the gut is rather immune tolerant whereby antigens can be recognized as food antigens or normal flora instead of eliciting protective immune responses (Mutwiri et al., 2005; Ilan, 2009). Therefore, vaccines to be administered orally have to overcome several barriers including significant dilution and dispersion in the GIT; competition with various live replicating bacteria, viruses, inert food and dust particles; enzymatic degradation; and low pH before reaching the target immune cells (Yeh et al., 1998).

At present, there are limited number of oral vaccines licensed for human use including polio, cholera, typhoid, rotavirus and adenovirus vaccines (Ogra et al., 2001; Aziz et al., 2007). Most of these available oral vaccines are composed of attenuated live microorganisms that can replicate in the mucosa and elicit a sustained immune response (Vajdy et al, 2004). Furthermore, residual toxicity by inactivation that might be present and possible induction of disease via reversion or mutation to an infectious agent to people with impaired immune response following administration of attenuated live vaccines, has changed the focus on vaccine development (Ryan et al., 2001; Perrie, 2006).

Therefore, efforts have focused on the development of subunit vaccines (nonreplicating antigens) that can be delivered orally by various delivery systems. Sub-unit vaccines are reported to have poor immunogenic response than traditional vaccines although they offer a much safer alternative (Lavelle, 2005). Traditional vaccines produce strong immunogenic response because often contain many components, partly reactogenic contaminants such as bacterial DNA, RNA or LPS in whole cell vaccines that can elicit additional T cell help or function as adjuvants, whereas in sub-unit vaccines, some of these components have been eliminated (Gupta and Siber., 1995; Moingeon et al., 2002; Wack and Rappuoli, 2005; Petrovsky, 2008). However, the use of adjuvant systems has
proven to enhance the immunogenicity of these sub-unit vaccines through protection (i.e. prevent degradation of the antigen in vivo) and enhanced targeting of the antigens to professional antigen-presenting cells and therefore calls for more development of vaccine adjuvants (Brayden, 2001; Perrie et al., 2008; Harandi et al., 2010).

Therefore, in order to develop oral vaccines, two important features would be required:
(1) Appropriate delivery systems that would protect the antigens to be delivered orally from the harsh environment of the gastrointestinal tract (GIT) (e.g. acid proteolytic enzymes, bile, temperature, and mucus), as well as enabling antigen uptake from the GIT, and antigen-presentation to appropriate cells of the immune system for the generation of desired immune responses
(2) Potential adjuvants which can stimulate the immune system to initiate appropriate immune responses against the delivered antigens (Zhou and Neutra, 2002; Simerska et al., 2009).

3.7.1 Roles of adjuvant

When conjugated to vaccines, adjuvants can enhance the immunogenicity of highly purified or recombinant antigens; can reduce the amount of antigen or the number of immunizations needed for protective immunity; can improve the efficacy of vaccines in newborns, the elderly or immune-compromised persons and can be used as antigen delivery systems for the uptake of antigens by the mucosa (Gupta and Siber., 1995; Petrovsky and Aguilar, 2004; Aguilar and Rodriguez. 2007; Rajput et al., 2007).

Adjuvants can be broadly separated into two categories based on their principal mechanisms of action:

(i) Particulate antigen delivery systems, (e.g., emulsions, microparticles, Immunostimulating complexes (ISCOMs), virus-like particles (VLPs) and liposomes) and function mainly to target associated antigens into antigen-presenting cells.
(ii) Immunostimulatory adjuvants derived predominantly from pathogens and often represent pathogen-associated molecular patterns (e.g., lipopolysaccharide, monophosphoryl lipid A, CpG DNA), which activate cells of the innate immune system (Gupta and Siber, 1995; Singh and O’Hagan, 2002; Moingeon et al., 2002).

Several authors have reported different classes of adjuvants that have been evaluated for enhancing immune responses to vaccines which are summarized in Table 3.2 (Gupta and Siber., 1995; Chen and Langer, 1998; Singh and O’Hagan, 2002; Moingeon et al., 2002; Zhou and Neutra, 2002; Rajput et al. 2007; Petrovsky, 2008).

**Table 3.2**: Examples of Different Classes of Adjuvants That Have Been Evaluated for Enhancing Immune Responses to Vaccines

<table>
<thead>
<tr>
<th>Classification</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MINERAL SALTS</strong></td>
<td>Aluminum salts (hydroxide, phosphate, alum)</td>
</tr>
<tr>
<td></td>
<td>Calcium phosphate</td>
</tr>
<tr>
<td><strong>IMMUNOADJUVANTS</strong></td>
<td>Cytokines e.g. IL-2, IL-12,</td>
</tr>
<tr>
<td></td>
<td>Granulocyte-macrophage colony stimulating factor (GM-CSF)</td>
</tr>
<tr>
<td><strong>EMULSIONS</strong></td>
<td>MF59 (stabilized squalane/sater)</td>
</tr>
<tr>
<td></td>
<td>QS21 (purified saponin from barks of Quillaja Saponaria tree)</td>
</tr>
<tr>
<td></td>
<td>Incomplete Freund adjuvant (IFA), without the killed mycobacteria</td>
</tr>
<tr>
<td><strong>NATURAL/SYNTHETIC BACTERIAL PRODUCTS</strong></td>
<td>Monophosphoryl lipid A (MPL)</td>
</tr>
<tr>
<td></td>
<td>RC-529 (synthetic MPL-like acylated monosaccharide)</td>
</tr>
<tr>
<td></td>
<td>Holotoxins (cholera toxin (CT); Lymphotoxin (LT) from Escherichia coli, Pertussis toxin (PT)</td>
</tr>
<tr>
<td></td>
<td>Bacterial DNA, cytidine-phosphate-guanosine (CpG) oligonucleotides`</td>
</tr>
<tr>
<td><strong>MICROPARTICLES/PARTICULATE ADJUVANTS</strong></td>
<td>Liposomes</td>
</tr>
<tr>
<td></td>
<td>Virosomes</td>
</tr>
</tbody>
</table>
Microparticles offer various significant advantages as drug delivery systems, including:

(i) An effective protection of the encapsulated active agent against (e.g. enzymatic) degradation.

(ii) The possibility to control the release rate of the incorporated drug over periods of time and (iii) an easy administration (O'hagan, 1996; Siepmann and Siepmann, 2006).

### 3.7.2 Oral vaccine delivery systems

Delivery of a vaccine via the oral route in a sufficient dose to induce a protective immune response depends on overcoming the loss of antigen integrity that occurs during intestinal passage. Several strategies have been employed to prevent this loss of antigenicity and improve the delivery of vaccines by the oral route. These include live vectors, plant-based edible vaccines system, and particulate formulations such as microparticles, liposomes, and virus-like particles (Litwin et al., 1997; Ellis, 2001; Moingeon et al., 2002; Lavelle, 2005). Enteric coated vaccine systems have also been explored as another strategy for delivering oral vaccines by protecting the vaccine from harsh environment of the GIT and release the delivered vaccine in unencapsulated form in the intestine following dissolution of the enteric polymer film (Jain et al., 1996; Snoeck et al., 2003; Huyghebaert et al., 2005).

In order for an effective immune response to be mounted against orally administered antigens, the antigen must be taken up and presented to the immune system through M cells of the Peyer’s patches present in the small intestine (Figure 3.7) that function to sample the intestinal contents and present them to underlying gut-associated lymphoid tissue (GALT) (Chen and Langer, 1998; Clark et al., 2001; Lavelle, 2005; SSilin et al.)
The GALT consist of inductive sites (where antigens are encountered and responses are initiated) and effector sites (where local immune responses occur) and is linked by a homing system, whereby cells induced by antigen in the GALT migrate to the circulation and, subsequently, colonize the mucosa and thus induction of immune response locally in the gut and at distant mucosal sites, as well as systemic humoral and cellular immune responses by oral vaccination (Yeh et al., 1998; Clark et al., 2001; Lavelle, 2005).

For the successful delivery of antigens by the targeted oral vaccine carrier, a number of considerations have to be taken into account as reported by many authors. To mention few but not exhaustive, these include protection of antigens from intestinal metabolism, high entrapment efficiency in particulate formulation, stability of antigen retained in the particle, uptake of vaccine by M cells through targeting and/or adjuvants and particle size (Table 3.3) of carrier system (O'hagan, 1996; Thomas et al., 1996; Yeh et al., 1998; Brayden et al., 2005).

Table 3.3: Sites, mechanisms and particle sizes proposed to be involved in the uptake of particles.

<table>
<thead>
<tr>
<th>Site</th>
<th>mechanism</th>
<th>Particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villus tips</td>
<td>persorption</td>
<td>5-150 µm</td>
</tr>
<tr>
<td>Intestinal macrophages</td>
<td>phagocytosis</td>
<td>1 µm</td>
</tr>
<tr>
<td>Enterocytes</td>
<td>endocytosis</td>
<td>&lt; 200 nm</td>
</tr>
<tr>
<td>Peyer’s patches</td>
<td>transparacellular</td>
<td>&lt; 10 µm</td>
</tr>
</tbody>
</table>
3.8 Materials and Methods

Presented in this chapter are the laboratory methods carried out for the extraction and isolation of yeast glucan from baker’s yeast cells (Saccharomyces cerevisiae), followed by encapsulation to form cationic yeast carriers, antigen loading into yeast carriers, formulation of pellets and minitablets containing antigen loaded in yeast carriers/shells (yeast-glucan) and enteric coating of pellets and characterization of these dosage forms.

Materials and equipment used for the extraction and purification of yeast-glucan from baker’s yeast cells (BRUGGEMAN instant) are listed in Table 3.4a and 3.4b.

**Table 3.4a: Materials used for the extraction and purification of baker’s yeast cells**

<table>
<thead>
<tr>
<th>Name of material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker’s yeast cells, (<em>Saccharomyces cerevisiae</em>), BRUGGEMAN instant</td>
<td>Algist Bruggeman N.V. Gent – Belgium</td>
</tr>
<tr>
<td>Sodium hydroxide pellets</td>
<td>VWR® International, Leuven.</td>
</tr>
<tr>
<td>Hydrochloric acid 37%</td>
<td>VWR® International, Leuven.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Pharm-tech lab. Ghent univ.</td>
</tr>
<tr>
<td>Disinfectol® solution (Ethanol denaturated with up to 5% Ether)</td>
<td>Chem-Lab, Belgium</td>
</tr>
<tr>
<td>Acetone</td>
<td>LAR BLOEI, Belgium</td>
</tr>
</tbody>
</table>
Table 3.4b: Equipment used for the extraction and purification of baker’s yeast cells

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass bottle, 2000ml</td>
<td>SCHOTT, Germany</td>
</tr>
<tr>
<td>Balance</td>
<td>Mettler-Toledo, Switzerland</td>
</tr>
<tr>
<td>Heater stirrer</td>
<td>Heidolph, Germany</td>
</tr>
<tr>
<td>Magnetic stirrer</td>
<td>LABINCO, Belgium</td>
</tr>
<tr>
<td>Thermometer, 0-200°C</td>
<td></td>
</tr>
<tr>
<td>pH meter</td>
<td>Consort, Belgium</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Heraeus, Germany</td>
</tr>
</tbody>
</table>

3.8.1 Extraction and purification of yeast-glucan from Baker’s yeast, (Saccharomyces cerevisiae).

Extraction and purification of yeast-glucan from baker’s yeast cell walls (Saccharomyces cerevisiae) was carried out according to the reported procedure for isolating fungal-derived β-D-glucans involving treatment with hot alkali and acids (Bacon et al. 1969; Sandula et al., 1999; Soto and Ostroff, 2008).

One liter solution of one molar Sodium hydroxide (1M NaOH) was prepared by dissolving 40g of sodium hydroxide pellets and stirred at 80°C. One hundred gram of Baker’s yeast (Saccharomyces cerevisiae) (BRUGGEMAN instant) was then suspended into this preheated solution and the reaction mixture was continuously stirred gently and maintained at a temperature of 80°C for 1 hour (Figure 3.9).
The mixture was centrifuged at 2000 x g for 10 minutes to collect insoluble material containing yeast cell wall (shells) components. The centrifuged insoluble material was then suspended in 1 liter of distilled water and its pH was corrected from 12 -13 to 4-5 using conc. HCl.

**Figure 3.9:** Preheating of 1M Sodium Hydroxide solution and Baker’s yeast cells (BRUGGEMAN instant) for extraction process.

The reaction mixture was allowed to heat up to 55°- 60°C and incubated/maintained at this temperature for 1hour (Figure 3.10).

**Figure 3.10:** Incubation of yeast cell-wall extracts in water at 55°- 60°C with adjusted pH 4-5.

The obtained suspension was centrifuged at 2000x g for 10 minutes to collect insoluble cell-wall material (sediment/precipitate) and the supernatant was discarded. The sediment was washed once with one liter of distilled water, and the precipitate was collected by centrifugation at 2000x g for 10min. The precipitate was again washed thrice with 800ml
of Disinfectol® solution (ethanol denaturated with up to 5% ether) and the sediment collected by centrifugation at 2000x g for 10min.

The sediment was dried in an oven at 40°C for 24hr. After grinding in a mortar and pestle, the yield was 9.45g of fine slightly off-white powder or 9.45% of the total weight of the yeast cells used and was designated as yeast-glucan shells or simply yeast shells (Figure 3.11).

**Figure 3.11**: Extracted dried yeast cell-wall components (yeast-glucan shells).

**Figure 3.12**: Schematic overview of the extraction and isolation of yeast-glucan from Baker’s yeast cell walls.
3.8.2 Antigen loading into yeast carriers

3.8.2.1. Preparation of cationic yeast carriers

Yeast-glucan cationized carriers were developed using layer-by-layer (LBL) assembly of yeast-glucan shells suspended and held together by electrostatic interactions between polyelectrolyte solutions of anionic charged tRNA genetic material and cationic charged Polyethylenimine (PEI) polymer, each separately dissolved in Tris HCL-EDTA-NaCl (TEN) buffer solution (Soto and Ostroff, 2008). Materials used for the preparation of Cationic yeast carriers are listed in Table 3.5.

Table 3.5: Materials used for the preparation of Cationic yeast carriers

<table>
<thead>
<tr>
<th>Name of material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIZMA® HYDROCHLORIDE (Tris [hydroxymethyl] aminomethane hydrochloride)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Polyethylenimine (PEI) polymer, water-free branched, (25kDa)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Ribonucleic acid from Torula utilis yeast, (tRNA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid disodium salt hydrate (EDTA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>VWR® International, Leuven</td>
</tr>
</tbody>
</table>

In order to form cationic yeast carriers that could allow antigen loading, the following solutions were preliminarily prepared so that the yeast shells could be suspended in during layer-by-layer formation.
3.8.2.2. Preparation of Buffer solution, Tris HCL-EDTA-NaCl (TEN) solution

7.88g of Tris HCl (Mw. 157.6), 0.744g of EDTA (Mw. 372); and 8.766g of NaCl (Mw. 58.44) were weighed and dissolved in distilled water to produced 1 liter buffer solution with 50mM Tris HCl, 2mM EDTA, and 0.15mM NaCl (TEN) concentration. The pH of TEN buffer solution was adjusted to pH 8.0 using 1M NaOH solution.

3.8.2.3. Preparation of anionic core polymer tRNA solution

1g of tRNA was weighed and dissolved in 100ml of buffer TEN solution to make 10mg/ml anionic core polymer tRNA in TEN.

3.8.2.4. Preparation of cationic PEI (Polyethylenimine) solution

200mg of PEI was weighed and dissolved in 100ml of buffer TEN to make 2mg/ml cationic PEI in TEN.

3.8.2.5. Preparation of cationic yeast carriers

10g of yeast shells was mixed with 50ml of the anionic core polymer tRNA (10mg/ml in TEN) to minimally hydrate the powder particles. The resultant slurry was incubated for 2 hours at room temperature to allow the particles to swell and adsorb the tRNA solution. Sufficient amount of cationic PEI (2mg/ml in TEN), approx. 150 ml, was added to form yeast-glucan shells encapsulated tRNA polypelexes, and the particles were resuspended by homogenization/stirring for 1h to allow PEI adsorption and microcomplex formation. Sufficient distilled water was added to the resultant suspension to obtain approximately 500 ml. This mixture was centrifuged at 2000 x g for 10 minutes and supernatant discarded.
The washing procedure with distilled water was repeated three times each at a centrifugation speed of 2000 x g for 10 minutes resulting into the collection of sediment of yeast-glucan cationized carriers and supernatant discarded.

**Figure 3.13**: Schematic overview of the preparation of cationic yeast-glucan carriers.

### 3.8.2.6. Loading of Bovine Serum Antigen to Cationic yeast carriers

Cationic yeast-glucan carriers were loaded with Bovine Serum Albumin (BSA), chosen as the model protein antigen in this work in the ratio of 1:1; i.e. 1g of BSA was loaded with 1g of cationized yeast-glucan. The following were the procedures carried out to load the BSA antigen into cationic yeast-glucan carriers.

#### 3.8.2.6.1. Preparation of Phosphate-buffered Saline (PBS buffer- 7.4 pH)

Phosphate-buffered Saline was prepared by dissolving the constituents in one liter of distilled water and its pH adjusted to 7.4 with 1M NaOH solution as listed in Table 3.6. The buffer was used as a diluent for cationic yeast-glucan carriers and BSA.
Table 3.6: Composition of Phosphate-buffered Saline (PBS buffer- 7.4 pH)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride, (NaCl)</td>
<td>8.0</td>
</tr>
<tr>
<td>Potassium Chloride, (KCl)</td>
<td>0.2</td>
</tr>
<tr>
<td>Disodium Hydrogen Phosphate, (Na$_2$HPO$_4$)</td>
<td>1.44</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate, (KH$_2$PO$_4$)</td>
<td>0.24</td>
</tr>
<tr>
<td>Distilled water to</td>
<td>1.0</td>
</tr>
<tr>
<td>pH (adjusted with 1M NaOH) to</td>
<td>7.4</td>
</tr>
</tbody>
</table>

3.8.2.6.2. Loading of BSA to cationic yeast carriers in PBS solution

Ten gram of BSA antigen was dissolved in one liter of PBS (pH 7.4) and stirred for five minutes to form a mixture of BSA/PBS buffer solution. This was followed by the addition of ten gram of cationic yeast carriers to this buffer solution containing BSA, then, stirred for thirty minutes and incubated overnight in a refrigerator. The mixture was centrifuged at 2000 x g for ten minutes to collect sediment of loaded BSA/yeast-glucan.

The collected sediment (loaded BSA/ yeast-glucan) was washed thrice with distilled water (approx. 800ml) to remove traces of PBS solution and the precipitate was again collected by centrifugation at 2000 x g for ten minutes. The precipitate was finally washed thrice with acetone (approx. 200ml) and the sediment collected by centrifugation at 2000 x g for ten minutes. The sediment was dried in an oven at 25°C for 24 hr forming dry granules which were then grinded using mortar and pestle to get loaded BSA/yeast-glucan carriers or loaded BSA/yeast carriers.
Figure 3.14: Schematic overview of the loading of cationic yeast-glucan carriers with BSA.

3.8.3 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was used to visualize microscopically the uptake and attachment of fluorescent labeled ovalbumin (OVA) (used as a model antigen) to yeast shells.

100 mg of yeast shells was diluted with 500µl of tRNA solution (10mg/ml in TEN buffer) and vortexed well for 1 minute to form a slurry which was incubated for 30 minutes at room temperature. A small sample of the slurry was scooped to serve as a control sample before addition of cationic PEI solution. The remaining slurry was diluted with 5 ml of cationic PEI (2mg/ml in TEN buffer) to form a suspension and vortexed again for 1 minute, followed by an incubation period of 30 minutes at room temperature.
The resulting suspension was centrifuged at 2000 x g for 10 minutes to collect the sediment and washed twice with demineralized water. Loading with OVA was performed by adding 10ml of OVA solution (10mg/ml in PBS) followed by thorough mixing and then mounted on the CLSM.

3.8.4. Formulation of fast-disintegrating pellets loaded with Bovine serum antigen (BSA/yeast-glucan pellets)

3.8.4.1. Pellet Production

Pellets loaded with 5% w/w BSA/yeast-glucan were produced via the extrusion/spheronization technique. Enzyme resistant starch – type III, modified starch grade (UNI-PURE® EX starch, National Starch and Chemical Co., Bridgewater, NJ, USA) was chosen as the main spheronizing aid (DukiC-Ott et al., 2008). Other ingredients incorporated into the formulation were: hydroxypropylmethylcellulose (HPMC) (Methocel® E15) as a binder, sorbitol to improve wettability in the formulation and distilled water as granulating liquid.

Loaded BSA/yeast-glucan carriers, UNI-PURE®EX starch, Methocel® E15 and sorbitol were dry mixed (batch size: 300g) for 15 minutes using planetary mixer (Kenwood Chief, Hampshire, UK) with a K-shaped mixing arm to obtain a uniform powder blend. 230g of distilled water was added at once to the powder mixture in the same mixer and blended for 5 minutes to form a wet granulated mass ready for extrusion. The wet mass was extruded axially at a speed of 50 rpm using a single screw extruder (Dome extruder, Lab model DG-L1, Fuji Paudal, Tokyo, Japan) fitted with dome-shaped screen of 1mm diameter perforations and 1.2mm thickness. The formed extrudates were spheronized for 5 minutes in a spheronizer (Caleva Model 15, Caleva, Sturminster Newton, Dorset, UK) with a cross-hatched friction plate at a speed of 850 rpm to form pellets followed by drying in an oven at 40°C for 24hrs.
Table 3.7: Composition of 5%w/w BSA/yeast-glucan Pellets

<table>
<thead>
<tr>
<th>Name of Ingredient</th>
<th>Quantity (g)</th>
<th>Trade name &amp; supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loaded BSA/ yeast-glucan carriers</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Modified (resistant) starch</td>
<td>241.965</td>
<td>UNI-PURE® EX starch; National Starch &amp; chemical co., Bridgewater, New Jersey, USA</td>
</tr>
<tr>
<td>Hydroxypropylmethylcellulose</td>
<td>13.965</td>
<td>Methocel® E15 LV EP Colorcon, Dartford, UK</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>29.1</td>
<td>Sorbidex® P 16616; Cerestar, Vilvoorde, Belgium</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>230.0</td>
<td></td>
</tr>
</tbody>
</table>

As release of BSA from the pellets can not be measured spectrophotometrically, we also made riboflavin (5%w/w) loaded pellets. These “tracer pellets” were coated in the same batch as the BSA loaded pellets, and afterwards dissolution tests were performed with the riboflavin pellets in order to evaluate coating efficiency.

Table 3.8: Composition of 5%w/w riboflavin sodium phosphate pellets

<table>
<thead>
<tr>
<th>Name of Ingredient</th>
<th>Quantity (g)</th>
<th>Trade name &amp; Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin sodium phosphate</td>
<td>15.0</td>
<td>Sigma-aldrich</td>
</tr>
<tr>
<td>Modified (resistant) starch</td>
<td>241.965</td>
<td>UNI-PURE® EX starch; National Starch &amp; chemical co., Bridgewater, New Jersey, USA</td>
</tr>
<tr>
<td>Hydroxypropylmethylcellulose</td>
<td>13.965</td>
<td>Methocel® E15 LV EP Pharm; Colorcon, Dartford, UK</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>29.1</td>
<td>Colorcon, Dartford, UK</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>230.0</td>
<td></td>
</tr>
</tbody>
</table>

3.8.4.2. Coating of pellets with Eudragit® 30L D-55, dispersion 30%w/w.

A total weight of 400g pellets (300g riboflavin pellets and 100g of BSA/yeast-glucan pellets) of size fraction 710 - 1400 µm were mixed together and coated in a fluid bed
coating equipment (GPCG1, Glatt, Binzen, Germany) using a bottom spray mode with Würster insert.

**Table 3.9: Composition of the coating dispersion**

<table>
<thead>
<tr>
<th>Name of the ingredient</th>
<th>Quantity (g)</th>
<th>Function</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eudragit® 30L D-55, 30% w/w dispersion</td>
<td>357.0</td>
<td>Acid-resistant film forming polymer</td>
<td>Rohm, Darmstadt, Germany</td>
</tr>
<tr>
<td>Glyceryl monostearate</td>
<td>9.1</td>
<td>Glidant</td>
<td>Federa, Braine-l’Alleud, Belgium</td>
</tr>
<tr>
<td>Polysorbate 80 (33% aq.sol)</td>
<td>11.2</td>
<td>Wetting agent/surfactant</td>
<td>Alpha Pharma, Nazareth, Belgium</td>
</tr>
<tr>
<td>Triethyl citrate (TEC)</td>
<td>21.7</td>
<td>Plasticizer</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>301.0</td>
<td>Dispersion medium</td>
<td></td>
</tr>
</tbody>
</table>

**3.8.4.3 Preparation of coating dispersion (700g)**

11.2g of polysorbate 80 (33% aqueous solution), 21.7g of triethylcitrate and 301g of water were stir heated to 70-80°C (above the melting point of glycerol monostearate). Then, 9.1g of glycerol monostearate was added. This mixture was removed from the heating source and homogenized for 10 minutes using a rotor-stator mixer (Silverson, Bucks, UK). Lastly, 357g of Eudragit® 30L D-55 was added and stirred gently with a magnetic stirrer for 30 minutes to homogenize and stabilize the dispersion before starting the coating process. The dispersion was constantly mixed gently with a magnetic stirrer throughout the coating process to maintain its homogeneity.

Pellets were pre-heated to 23-26°C prior to suspension spraying while the inlet air temperature was set between 28 and 30°C in order to maintain a product temperature of 25 - 26°C. The coating dispersion was maintained for 2hrs at a spray rate of 4.0 g/min,
through a 0.8 mm nozzle using an atomizing air pressure of 1.5 bars. Pellets were left to
dry at the same product temperature (26°C) for 15 min after coating. At the end of the
process, the total weight gain of dry coated pellets was 477g (equiv. to 19.25% w/w dry
polymer weight gain) from an initial weight of 400g.

3.8.5 Formulation of fast-disintegrating minitablets loaded with Bovine serum
antigen (BSA/yeast-glucan minitablets)

Minitablets of 5mm diameter loaded with 5%w/w BSA/yeast-glucan were prepared by
direct compression. Loaded BSA/yeast-glucan and required excipients (Table 3.10) were
dry mixed (batch size: 100g) for 15 minutes in a Turbula® mixer (model T2A, W.A.
Bachofen, Basel, Switzerland) to obtain a uniform powder mixture. The powder blend
was directly compressed into tablets with a compression force of 150-155kgf (kilogram-
force, measured by digital indicator, AD-4532A connected to the tablet press) using a
single punch eccentric tablet press type EKO, Korsch, Berlin, Germany.

Table 3.10: Composition of 5%w/w BSA/yeast-glucan minitablets

<table>
<thead>
<tr>
<th>Name of the ingredient</th>
<th>Quantity (g)</th>
<th>Function</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loaded BSA/ Yeast-glucan pulv</td>
<td>5.0</td>
<td>Active ingredient</td>
<td></td>
</tr>
<tr>
<td>Microcrystalline Cellulose, Avicel® PH 101</td>
<td>45.0</td>
<td>Binder</td>
<td>FMC, BioPolymer, Little Island, Ireland</td>
</tr>
<tr>
<td>Lactose, Pharmacon® DCL 21</td>
<td>45.0</td>
<td>Filler/diluent</td>
<td>Pharmacon</td>
</tr>
<tr>
<td>Cross-linked polyvinylpyrrolidone, Polyplasdone® XL-10</td>
<td>5.0</td>
<td>Super disintegrant</td>
<td>ISP, Switzerland</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>0.5</td>
<td>Lubricant</td>
<td>Fagron, Belgium</td>
</tr>
</tbody>
</table>
3.8.6 Evaluation and characterization of pellets and minitablets

3.8.6.1 Pellet size analysis

Sieve analysis method was used to evaluate pellet size and yield, whereby 250g pellets were sieved for 10 minutes at an amplitude of 3mm on a shaker (Type VE 1000, Retsch, Haan, Germany) using 1400, 1120, 1000, 800, 710, 500 and 250µm sieves (Retch, Haan, Germany) in order to remove fines and agglomerates. The pellet yield was calculated based on the pellet fraction between 710 - 1400µm separated by sieving and presented as a percentage of the total pellet weight. This size fraction was used for all other measurements.

3.8.6.2 Pellet shape and size analysis

An image analysis system was used to determine shape and size of coated pellets. Photomicrographs of pellets were taken with a digital camera (Camedia® C-3030 Zoom, Olympus, Tokyo, Japan), linked with a stereomicroscope system (SZX9 DF PL 1.5x, Olympus, Tokyo, Japan). A cold light source (Highlight 2100, Olympus, Germany) and a ring light guide (LG-R66, Olympus, Germany) were used to obtain top light illumination of the pellets against a dark surface. The images were analyzed by image analysis software (AnalySIS®, Soft Imaging System, Münster, Germany). The magnification was set in a way that one pixel corresponded to 5.7µm and around 100 pellets were analyzed from the prepared batch. Each individual pellet was characterized by the mean Feret diameter (FD) (average of 180 calliper measurements with an angle of rotation of 1°), aspect ratio (AR) (ratio of longest Feret diameter and its longest perpendicular diameter) and two-dimensional shape factor (eR), as described by Podczeck and Newton (1994).

\[ e_R = \frac{2 \cdot \pi \cdot r}{P_m} \cdot \sqrt{1 - \left(\frac{b}{l}\right)^2} \]  

(equation. 3.1)
Where \( r \) is the radius, \( P_m \) is the perimeter, \( l \) is the length (longest Feret diameter) and \( b \) is the breadth (longest diameter perpendicular to the longest Feret diameter) of a pellet. An average value for all pellets was calculated as the mean pellet size (mean FD).

### 3.8.6.3 Pellet friability test

10g of pellets (FS, 10g) was placed in an abrasion wheel together with 200 glass beads (diameter: 4mm) and fitted to a friabilator; (type PTF, Pharma Test, Hainburg, Germany). The sample was subjected to falling shocks for 10 minutes at a rotational speed of 25 rpm and fines collected by sieving through 250\( \mu \)m mesh for 5 minutes at 2mm amplitude. The weight difference was obtained by weighing the pellets retained above 250\( \mu \)m (Fa) and compared to the initial weight of the sample and percentage of friability determined using the following equation:

\[
Friability(\%) = \left( \frac{F_s - F_a}{F_s} \right) \times 100
\]  
(equation. 3.2)

### 3.8.6.4 Pellet disintegration test

The disintegration time for uncoated and coated loaded yeast-glucan pellets was measured in a disintegrator (Type PTZ, Pharma Test, Hainburg, Germany) using a method modified from the Eur. Ph. 6\(^{th}\) edition monograph for tablet disintegration. Using a 500 \( \mu \)m mesh placed at the bottom of each tube (6 tubes) and discs to increase the mechanical stress on the pellets, 100mg of uncoated pellets and coated pellets were dispersed in each tube separately and immersed in a beaker containing 600ml of demineralized water for uncoated pellets and phosphate buffer (PB pH 6.8) for coated pellets respectively as disintegration media (both preheated to 37°C). Results represent the average of six determinations.
3.8.6.5 Dissolution tests

The dissolution tests were performed using the USP apparatus (VK 8000, VanKel, New Jersey, USA) with paddles at a rotational speed of 100 rpm, in 900 mL dissolution medium at 37°C. Acidic dissolution medium (0.1N HCl; pH 1.2) was used during the first 2h, followed by 1h in phosphate buffer (PB; pH 6.8). During dissolution in 0.1N HCl, samples of 5 mL were withdrawn from the dissolution vessel at 5, 10, 15, 20, 30, 45, 60, 75, 90, 105 and 120 min and in PB (pH 6.8) at 5, 10, 15, 20, 30, 45 and 60 min. As mentioned before, the dissolution test was performed with the riboflavin loaded pellets and the sample amount used for analysis (1g) was adjusted to obtain sink conditions. The concentration of riboflavin was measured spectrophotometrically at 267 nm in 0.1N HCl and pH 6.8 PB, respectively, by means of a double-beam spectrophotometer (UV-1650PC, Shimadzu, Kyoto, Japan).

According to the requirements from Eur. Ph. 6th edition, an enteric coat was successfully applied if less than 10% of drug is released after 2 h of dissolution in acid dissolution medium (0.1N HCl).

3.8.6.6 Scanning electron microscopy

The morphology of the coating surface and the coating thickness of pellets were visualized by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan). Pellets were radially sheared to obtain cross-sections and coated with platinum using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan) to assure conductivity. Photomicrographs were taken with a scanning electron microscope and the coating thickness of a pellet was measured at four sites per pellet.

3.8.6.7 Tablet hardness test

The resistance to crushing of the tablets (n=20) was determined as described in European Pharmacopoeia, 6.0th edition, using an automated hardness tester, SOTAX HT 10 V1.31,
equipped with auto alignment device and software (Q-Doc hardness tester software) for statistical processing. The test jaws constant speed was 1.00 mm/sec. Results were expressed as the mean, minimum and maximum values of the forces measured, all expressed in Newtons representing twenty determinations.

3.8.6.8 Tablet friability test

The friability test for BSA/yeast-glucan minitablets was determined according to Eur. Ph. 6th edition, monograph for friability of uncoated tablets.

A sample of twenty tablets (Ws) was weighed and placed on a sieve 1000 µm size to remove any loose dust with the aid of air pressure before being placed in a friability drum and fitted to a friabilator (type PTF, Pharma Test, Hainburg, Germany). The sample was subjected to roll and fall by rotating the drum 100 times and then followed by removal of any loose dust generated from the tablets during the test. The tablets were weighed again to determine their final weight (Wf). The friability was expressed as the loss of mass and was calculated as a percentage of the initial mass using the following equation:

\[
Friability(\%) = \left[ \frac{(W_s - W_f)}{W_s} \right] \times 100
\]  
(equation. 3.3)

3.8.6.9 Tablet disintegration test

The disintegration time for the minitablets was measured in a disintegrator (Type PTZ, Pharma Test, Hainburg, Germany) using a method from the Eur. Ph. 6th edition monograph for tablet disintegration. One tablet was placed in each of the six tubes and discs were added to each tube in order to increase the mechanical stress on the tablets. The assembly was suspended in a beaker containing 600ml of demineralized water as disintegration medium preheated to 37°C. Results represent the average of six determinations.
3.9 Results and Discussion

3.9.1 Laser confocal microscopy

Microscopic observation using confocal scanning laser microscopy showed a successful binding or entrapment of green-labeled fluorescent ovalbumin (OVA) antigens to yeast shells upon mixing anionic tRNA and cationic PEI solution (Figure 3.15) as a result of electrostatic interactions (Li et al., 2008; Soto and Ostroff, 2008).

The Confocal microscopy illustrated how the modification of the yeast shells with tRNA and cationic PEI allowed a successful loading of the shells with green fluorescent Ovalbumin (OVA).

![Image of yeast shells and green fluorescent ovalbumin](image.png)

**Figure: 3.15:** Confocal microscopy images showing the interaction between green fluorescent ovalbumin and yeast shells before and after cationic modification. The bar corresponds to 20µm. The left column is the green fluorescence image; the middle column is the transmission image and the right column is the overlay of both green fluorescence and transmission image.

The first row of Figure 3.15 shows no accumulation of green fluorescence inside the yeast shells as large excess of green fluorescence is observed in the solution outside the
yeast shells. Upon modification of yeast shells with tRNA, an identical situation is observed as shown in the middle row of Figure 3.15.

Contrary, after modification with tRNA and cationic PEI, the green fluorescent OVA strongly accumulated within the yeast shells leaving the external solution dark as shown in the lower row of Figure 3.15.

3.9.2 Sieve analysis

The size and weight distribution of sieved pellets (250g) are presented in Table 3.11, indicating that a pellet yield (710-1400 µm fraction) of higher than 90% could be obtained. This shows that the addition of a binder (hydroxypropylmethylcellulose) to UNI-PURE® EX starch was necessary to obtain an acceptable yield since the binder increased the mechanical strength of wet extrudates and consequently fewer fines were formed during spheronization (Dukic-Ott, et al., 2007a).

Table 3.11: Results of size and weight distribution of pellets by sieve analysis

<table>
<thead>
<tr>
<th>Sieve size fraction</th>
<th>Weight of sieved pellets (g)</th>
<th>Percentage yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 2000µm</td>
<td>1.000</td>
<td>0.4</td>
</tr>
<tr>
<td>1400-2000 µm</td>
<td>10.605</td>
<td>4.24</td>
</tr>
<tr>
<td>1120-1400 µm</td>
<td>64.788</td>
<td>25.92</td>
</tr>
<tr>
<td>1000-1120 µm</td>
<td>75.059</td>
<td>30.02</td>
</tr>
<tr>
<td>800-1000 µm</td>
<td>86.881</td>
<td>34.75</td>
</tr>
<tr>
<td>710-800 µm</td>
<td>7.481</td>
<td>2.99</td>
</tr>
<tr>
<td>500-710 µm</td>
<td>3.781</td>
<td>1.51</td>
</tr>
</tbody>
</table>
### 3.9.3 Pellet shape and size analysis

The shape of the pellets is characterized by the aspect ratio which describes the deviation in shape from a circle to an ellipse and by the roundness. The latter is an indication of surface irregularities. The image analysis results indicated that coated pellets (710-1400µm) had a Feret mean diameter of 0.967±0.15mm, sphericity (aspect ratio, AR) of 1.2±0.25 and a two-dimensional shape factor (eR), 0.87±0.11 (n=100), falling within the limits set by Chopra et al. (2002) for optimum pellets shape and flow characteristics.

### 3.9.4 Pellet friability test

Starch-based pellets had a high mechanical strength, since friability values of less than 0.01% were obtained. A low friability indicates the pellets’ ability to withstand the shear forces during fluid bed coating.

### 3.9.5 Pellet disintegration test

The time taken for complete disintegration of uncoated pellets was 5-10 minutes and 10-15 minutes for the coated pellets in water and PB pH 6.8 respectively. This showed that modified starch is a good choice as the main excipient for the formulation of pellets with fast-disintegrating properties and consequently fast drug release in dissolution medium (Dukić-Ott et al., 2008).

Furthermore, photomicrographs of coated pellets taken with a digital camera (Camedia® C-3030 Zoom, Olympus, Tokyo, Japan), linked with a stereomicroscope system (SZX9 DF PL 1.5x, Olympus, Tokyo, Japan) showed that the Eudragit® 30LD-55 coated pellets
did not dissolve when placed in acidic medium but showed dissolution in alkaline medium (Figure 3.16). These results suggest that Eudragit® 30LD-55 is a good stabilizer as gastric acid-resistant polymer.

![Figure 3.16: Photomicrographs visualizing uncoated and coated BSA/yeast glucan pellets in acidic and alkaline medium. Time scale: Left column, t = 0 min, the start of the experiment i.e. when the acid and alkali were added to the pellets. Middle column, t = 5 min, disintegration and appearance of pellets after five minutes exposure. Right column, t=10 min, disintegration and appearance of pellets after ten minutes exposure.](image)

3.9.6 Pellet dissolution test

In-vitro drug release profile (Figure 3.17) showed that all riboflavin was released in 30 minutes in phosphate buffer (pH 6.8). Drug release was fast due to disintegration of UNI-PURE® EX starch as the main excipient which ensures fast exposure of the drug to the dissolution medium and also due to the inclusion of sorbitol in the formulation which has high solubility in water (Dukic-Ott, et al., 2007b).

In addition to that, the drug release profile showed that enteric coating was successfully applied to the riboflavin pellets since after 2h of dissolution in acid dissolution medium (0.1N HCl), less than 10 % drug was released which is according to the requirements in European Pharmacopoeia, 6th edition (maximally 10%). The release in 0.1N HCl after 2h (4.4 %) was below the limits indicated in the European Pharmacopoeia.
3.9.7 Scanning electron microscopy

SEM enabled a detailed visualization of the empty yeast-glucan shells (Figure 3.18). It is clear that the yeast-glucan shells have the form of small microparticles, and that the BSA loaded shells have the same shape as the empty yeast-glucan shells.

SEM micrographs of the pellets showed that the pellets were spherical in shape and that the surface of the pellets coated with Eudragit® 30LD-55 was smooth (Figure 3.18). A cross-sectional view of the pellets revealed a rigid structure with low porosity and a coating thickness of 38µm to obtain a gastroresistant effect (Figure 3.19).
Figure 3.18: SEM pictures showing yeast shells (yeast-glucan), BSA loaded yeast-glucan shells, uncoated BSA/yeast-glucan pellets, coated BSA/yeast-glucan pellets and cross-section (half-sliced) coated BSA/yeast-glucan pellets.

Figure 3.19: SEM picture of a cross-section of a pellet coated with 30% (w/w) Eudragit® 30LD-55, indicating coating thickness.
3.9.8 Tablet hardness test

The hardness of a tablet refers to a measure of the crushing force exerted under diametrical loading. The mean, minimum and maximum measured forces for the representative tablets tested were 45N, 24N and 57N respectively. The obtained values are considered acceptable in relation to this study since the minitablets were sufficiently strong enough to withstand shear forces during friability testing and disintegrated within the required time of 5 - 10 minutes since the hardness of a tablet affects its disintegration time, the harder the tablet, the longer it takes to disintegrate.

3.9.9 Tablet friability test

Friability refers to the tendency for the tablets to form dusts or to break-up/off when subjected to abrasion forces. It is the ability of the compressed tablet to avoid fracture and breaking apart during transport.

BSA/yeast-glucan minitablets had a high mechanical strength, since friability values of less than 1% was obtained (friability = 0.5%). The pharmacopeial limits consider a maximum loss of 1 percent of the mass of the tablets tested to be acceptable. This low friability indicates the tablets ability to withstand the shear forces when subjected to mechanical shock or attrition.

3.9.10 Tablet disintegration test

The time taken for the minitablets to achieve complete disintegration was 5 - 10 minutes. This showed that the composition of the formulation with the inclusion of lactose (which is water soluble) and crospovidone (a superdisintegrand which is able to absorb large amount of water and improve its penetration in the formulation and thus increase the extent and rate of swelling of the tablets) are good choice as excipients for the formulation of fast-disintegrating tablets leading to a fast release of the drug substance in dissolution medium (Kristensen et al., 2002).
3.10 Conclusion

Yeast shells, extracted from *S. cerevisiae* have been shown to possess the ability to bind/entrap antigens following tRNA/PEI cores formation by electrostatic interaction inside yeast shells. With the purpose to serve as an oral vaccine delivery system, the yeast shells were incorporated into pellets formulation prepared via extrusion/spheronization and minitablets prepared by direct compression respectively.

Due to pellet disintegration, fast dissolution of “tracer pellets” containing riboflavin was achieved (>80% drug release in 30 minutes) when using UNIPURE® EX starch as the main excipient in pellet formulations prepared via the extrusion/spheronization technique. Likewise, fast-disintegrating (<10 minutes) minitablets using crospovidone as superdisintegrant and lactose could also be prepared by direct compression. The disintegration time for both uncoated pellets (5 - 10 min) and minitablets (5 - 10 min) was nearly the same, predicting similar bioavailability of the two developed types of dosage forms when administered orally.

In conformity with data reported in the literature, this study has demonstrated that yeast-glucan shells can bind/entrap antigens. The antigen-loaded yeast carriers when formulated into enteric coated pellets and minitablets, can then be safely delivered to macrophages and dendritic cells, thus, facilitating oral receptor-targeted (β-glucans receptors) delivery of antigens.

Though in-vivo studies to evaluate the performance of the prepared delivery systems (pellets and minitablets) are pending, the available data regarding the adjuvanticity of yeast-glucan show that, it is a promising candidate for vaccine delivery system to induce both mucosal and systemic immune responses.
References


http://www.beta-glucan.info/index.htm (cited on 7-2-2010)


http://www.clinicalimmunity.com/tumors.html (cited on 1-4-2010)