P-075	**Nanoencapsulation of bovine glycomacropeptide for food and biopharmaceutical applications.**

Balcão V.M.[1,2*,#]; Morsy T.A.[1]; Costa C.I.[1]; Matos C.M.[1]; Moutinho, C. G.[1] and Teixeira J.A.[2]

[1] Bioeng. Biopharm. Chem. Res. Group (GIBQB-CIAGEB), University Fernando Pessoa, Porto, Portugal. [2] Institute Biotech. Bioeng. (IBB), CEB – Univ. of Minho, Braga, Portugal.

* Supervisor # vbalcao@ufp.edu.pt

INTRODUCTION AND OBJECTIVES

Glycomacropeptide (GMP) has a MW of ca. 8000 Da and has been proven to possess beneficial bioactive roles which include induction of satiety by stimulation of cholecystokinin release from intestinal cells (Keogh 2010), inhibition of platelet aggregation, support of beneficial intestinal LAB, ability to bind cholera and E. coli enterotoxins, inhibition of bacterial and viral adhesions, modulation of immune system responses, (Bezkorovainy 1979; Azuma 1984; Thomä-Worringer 2006), and reduction of dental caries (Schaafsma 2000; Aimutis 2004; Janer 2004). As most proteins, GMP is fragile and even small conformational changes may reduce its biological activity. The development of strategies that may permit structural and functional stabilization of GMP via nanoencapsulation may increase its applicability in the food and biopharmaceutical industries (Srinivas 2010), by protecting the bioactive peptide from denaturation by proteolysis and dilution effects. Recently, the stabilization of proteins and enzymes based on nanoencapsulation procedures has started to gain momentum, based on encapsulation of such macromolecules in a nanoemulsion matrix (Ragoonanan 2007; Rawat 2008) with an hydrophilic core: similarly to the stabilization mechanism of osmolytes, in nanoencapsulation the water activity is altered thus affecting the molecular motions of the proteins. Therefore, nanosized vesicles with an hydrophilic core may significantly increase the structural and functional stability of bioactive proteins for applications in the food and biopharmaceutical sectors. In this context, water-in-oil-in-water (W/O/W) (nano)emulsions, which are examples of multiple emulsions in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous (external) aqueous phase (Bibette 1999; Ficheux 1998; García-Fuentes 2002; Wang 2006; Hanson 2008; Dupeyrón 2009) have been proven to be suitable carriers for the simultaneous entrapment-stabilization of bioactive lactoferrin (Costa 2010). Nanosized emulsions are a class of stable emulsions composed of surfactant and oil suspended in water, which (reported) stability makes them extraordinary and often described as "approaching thermodynamic stability" (Kotyla 2008; Gutiérrez 2008). Oral delivery of (bioactive) proteins has been long identified as one of the main challenges in drug delivery science and (bio)technology. Nanoemulsions (NE), being non-equilibrium systems with a spontaneous tendency to separate into the constituent phases, may possess however a relatively high kinetic stability, even for several years (Gutiérrez 2008). Nano-emulsions are non-equilibrium systems, and so energy input, generally from mechanical devices or from the chemical potential of the components, is required for their formation. In the present research work, bovine GMP was entrapped in the hydrophilic core contained within lipid nanovesicles, integrating W/O/W multiple NE, aiming at mimicking the multifunctional design of biological membranes, with several lipid matrices, and stabilizing layer compositions, with the ultimate goal of achieving its structural and functional stabilization. Due to their compartimentalized internal structure, multiple emulsions present advantages over simple O/W emulsions for encapsulation, such as the ability to carry both polar and non-polar molecules, and a better control over releasing of therapeutic molecules (Pays 2002; Davis 1987; Hanson 2008).

MATERIALS AND METHODS

Preparation of multiple GMP-encasing NE.

Production of multiple W/O/W NE with nanoencapsulated GMP was carried out in an UltraTurrax (T25D from IKA) under heating (ca 38 °C), with the bioactive GMP being previously dissolved in the (inner) aqueous phase (W) prior to being dispersed in the melted lipid (Softisan, from Sasol) during homogenization (10 min at 8000 rpm or 10000 rpm). Therefore, final W/O/W dispersion of GMP was obtained via a sequential (optimized) homogenization of a W/O dispersion involving two cycles for 10 min. The inner aqueous phase was constituted by HCl 10 mM, $CaCl_2$ 20 mM, Tween 80 and pure bovine GMP (from Davisco Foods); the intermediate oily phase encompassed glycerol, Softisan 100 and soybean phosphatidylcholine; finally, the outer aqueous phase encompassed Lutrol 68 (poloxamer 188 from BASF), ultrapure water and NaCl 10 mM. GMP-encasing nanovesicles were stored throughout 160 days under refrigerating conditions.

Evaluation of the combined effects of homogenization speed, GMP concentration and ionic strength of the inner aqueous phase for optimization of the nanoformulation (NF).

The combined effects of homogenization speed, GMP concentration and ionic strength of the inner aqueous phase were studied by producing emulsions at different homogenization speeds (from 8000 to 10000 rpm), with a

constant amount of GMP (50 mg) and ionic strengths ($CaCl_2$ 0.001 M, HCl 0.01 M) of the inner aqueous phase.

Determination of hydrodynamic size (HS) and Zeta potential (ZP).

The ZP and HS of the nanoemulsion particles were determined in a Zetasizer (model Nanoseries Nano-ZS) from Malvern Instruments.

RESULTS AND DISCUSSION

The mean size and size distribution (polydispersity index) were measured via Dynamic Laser Light Scattering. Buffered GMP was found to be more stable than unbuffered, purified GMP. Optimum homogenization time was found to be 10 min at 10000 rpm, since during such timeframe particle size was maintained at values ranging from 100 – 400 nm. Addition of an electrolyte (calcium chloride 0.001 M) to the inner aqueous phase proved to be most suitable by producing nanovesicles with a stable ZP (of ca. -10 mV) throughout storage time. However, when using a higher (0.1 M) electrolyte concentration, phase separation was notorious immediately after homogenization which can be correlated with the higher (stable and close to zero) values of ZP produced. Decreasing electrolyte concentration led to lower (more negative) and more stable values of ZP, since the electric charge of κ-casein GMP is highly negative in comparison with other milk proteins. Storage of the optimized multiple NE throughout 131 d at fridge temperature did not lead to observable phase separations, nor adhesion of nanovesicles to the container's wall.

CONCLUSIONS

A lipid with mild melting temperature was found most appropriate for the discontinuous oily phase. A homogeneization timeframe of 10 min, the use of an electrolyte with a low ionic strength, and an amount of GMP of 50 mg were found to be critical variables for producing stable nanovesicle dispersions with diameters ranging from 100-400 nm and ZP values of ca. -10 mV. The use of these multiple NE in formulating functional (bioactive) food formulations would possess inherent advantages due to the bioactivity of the naturally-occurring GMP, duly structural and functional stabilized via the nanoencapsulation procedure.

REFERENCES

- Keogh J.B. *et al.* (2010) *Effect of GMP fractions on cholecystokinin and food intake*, Br. J. Nutr. **8**: 1-5.
- Bezkorovainy A. *et al*. (1979) *Isolation of a Glycopolypeptide Fraction with Lactobacillus Bifidus Subspecies Pennsylvanicius Growth-Promoting Activity From Whole Human Milk Casein*, Am. J. Clin. Nutr. **32**: 1428-32.
- Azuma N. *et al*. (1984) *Bifidus Growth-Promoting Activity of a GMP Derived From Human K-Casein*, Agr. Biol. Chem. **48**(8): 2159-62.
- Thomä-Worringer C. *et al*. (2006) *Health effects and technological features of CMP*, Int. Dairy J. **16**: 1324-1333.
- Schaafsma G. *et al*. (2000) *Dairy ingredients as a source of functional foods*, Chapter 8, In: *Essentials of Functional Foods*, Schmidl, M. K. and Labuza, T. P. (Eds), pp. 181-204, Aspen Publishers Inc., USA, ISBN: 0-8342-1261-7.
- Aimutis W.R. (2004) *Bioactive Properties of Milk Proteins with Particular Focus on Anticariogenesis*, J. Nutr. **134**: 989S-95S.
- Janer C. *et al*. (2004) *The Effect of CMP and Whey Protein Concentrate on Streptococcus Mutans Adhesion to Polystyrene Surfaces and Cell Aggregation*, J. Food Qual. **27**: 233-238.
- Srinivas P.R. *et al*. (2010) *Nanotechnology research: applications in nutritional sciences*, J. Nutr. **140**: 119-124.
- Rawat M. *et al*. (2008) *Lipid carriers: a versatile delivery vehicle for proteins and peptides*, Yakugaku Zasshi (The Pharmaceutical Society of Japan) **128**: 269-280.
- Ragoonanan V. *et al*. (2007) *Protein stabilization*, Transfusion Medicine and Hemotherapy **34**: 246-252.
- Bibette J. *et al*. (1999) *Emulsions: Basic principles*, Rep. Prog. Phys. **62**: 969–1033.
- Ficheux M.F. *et al*. (1998) *Some stability criteria for double emulsions*, Langmuir **14**: 2702–2706.
- García-Fuentes M. *et al*. (2002) *Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules*, Colloids and Surfaces B: Biointerfaces **27**: 159-168.
- Wang Y.F. *et al*. (2006) *Structural evolution of polymer-stabilized double emulsions*, Langmuir **22**: 67–73.
- Hanson J.A. *et al*. (2008) *Nanoscale double emulsions stabilized by single-component block copolypeptides*, Nature **455**: 85-88.
- Dupeyrón D. *et al*. (2009) *Protein Delivery by Enteric Copolymer Nanoparticles*, J. Dispersion Sci. Tech. **30**:1188–1194.
- Kotyla T. *et al*. (2008) *Increased bioavailability of a transdermal application of a nano-sized emulsion preparation*, Int. J. Pharm. **347**: 144-148.
- Gutiérrez J.M. *et al*. (2008) *Nano-emulsions: New applications and optimization of their preparation*, Curr. Opinion Colloid & Interface Sci. **13** (4): 245-251.
- Pays K. *et al*. (2002) *Double emulsions: how does release occur?* J. Control. Release. **79**: 193-205.
- Davis S.S. *et al*. (1987) *Multiple emulsions as targetable delivery systems*, Methods Enzymol. **149**: 51–64.
- Hanson J.A. *et al*. (2008) *Nanoscale double emulsions stabilized by single-component block copolypeptides*, Nature **455**: 85-88.
- Costa, C. I., Morsy, T. A., Matos, C. M., Moutinho, C. G., Amorim, M., Pintado, M. E., Gomes, A. P., Teixeira, J. A., Balcão, V. M. (2010) *Nanoencapsulation of bovine LF for biopharmaceutical applications*, J. Nanoscience Nanotech. [submitted].