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**Thesis title:**

**Preparing, measuring and capturing  
G-protein coupled receptor (GPCR)  
signalling complexes for future  
development of cell-free assay technologies**

**Candidate: Ms Olgatina Bucco B.Biotechnology (Hons)**

**Academic Organisation Unit:**

**Department Medical Biochemistry, Faculty of Health Sciences,  
School of Medicine, The Flinders University of South Australia.**

**and**

**CSIRO Division of Molecular and Health Technologies**

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## SUMMARY

G-protein coupled receptors (GPCRs) are integral membrane proteins which represent primary cellular targets for intracellular signalling. Many of these receptors are altered in disease states and hence are the target for over 50% of marketed drugs. Despite their physiological importance, high-throughput, cell-free assays which measure functional or signalling activity are only recently being investigated. The current approach by the pharmaceutical industry to initially screen compounds for functionality is to use heterologous cell-based assay formats. The aim of this work was to reconstitute a cell-free GPCR signalling system on an appropriate platform (surface) as a prototype for future rapid drug screening and other applications. The proof-of-concept approach involved using the  $\alpha_{2A}$ -adrenergic receptor ( $\alpha_{2A}$ -AR) containing cell membrane preparations as the model GPCR, reconstituted with a set of heterotrimeric G-proteins;  $G\alpha_{i1}$  and  $\beta_1\gamma_2$  (the signal transducing complex being termed a “transductosome”). However, other receptors and G-proteins were also investigated. Receptors were initially obtained from natural (tissue) sources, however in the later stages they were expressed in a heterologous system (insect or mammalian expression system). G-proteins were expressed in *Spodoptera frugiperida* (*Sf9*) insect cells using the baculovirus expression system. Receptor expression was verified by radioligand binding assays and endogenous G-proteins were removed from membrane preparations using the chaotropic agent urea to allow for reconstitution with purified G-proteins. Signal transduction through the transductosome was measured using the [ $^{35}$ S]GTP $\gamma$ S binding assay. Receptor activated [ $^{35}$ S]GTP $\gamma$ S binding was used to determine functional reconstitution and to validate that the system was working in the normal physiological manner both on and off a surface (with surface attachment being via histidine attachment on the  $G\alpha_{i1}$  ( $_{6\times\text{HIS}}$ ) subunit). Using the captured (surface-attached) transductosomes, the  $IC_{50}$

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values for Rauwolscine, Yohimbine (potent  $\alpha_2$ -AR antagonists), Prazosin (potent  $\alpha_1$ -AR antagonist) and Propranolol ( $\beta$ -AR antagonist) displayed the appropriate rank order for this class of receptor. This cell-free, surface-attached signalling complex prototype may have use in the future development of drug screening and discovery assay technologies as well as other applications as an alternative to cell-based assays which are not readily amendable to miniaturisation, long term storage and therefore stable robust microarray formats.

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## **DECLARATION:**

‘I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.’

**Olgatina Bucco** (August 2005)

"Nobody climbs mountains for scientific reasons. Science is used to raise money for the expeditions, but you really climb for the hell of it." - Sir Edmund Hillary

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## PUBLICATIONS AND ABSTRACTS ARISING FROM THIS THESIS

<sup>#</sup>Leifert WR, Aloia, AL, **Bucco, O**, Glatz, RV and McMurchie, EJ (2005) G-protein coupled receptors in drug discovery: nano-sizing using cell-free technologies and molecular biology approaches. *J Biomol Screen*, Dec 10 (8) pp. 765-779.

<sup>#</sup>Leifert WR, Aloia, AL, **Bucco, O** and McMurchie, EJ (2005) GPCR-induced dissociation of G-protein subunits: G $\alpha$  and G $\beta\gamma$  subunit separation in early stage signal transduction. *Mol Membrane Biol*, Nov-Dec 22 (6) pp. 507-517.

**Bucco O**, Leifert WR, Aloia AL, Burnard SL, Barritt GJ and McMurchie EJ (2004) G-Protein receptor transduosome capture for cell-free, rapid drug screening *The Australian Health and Medical Research Congress Proceedings*, Poster presentation. **This poster was awarded the Johnson & Johnson prize for best translational research at the conference.**

**Bucco O**, Leifert WR, Aloia AL, Burnard SL, Barritt GJ and McMurchie EJ (2004) G-Protein receptor transduosome capture for cell-free, rapid drug screening. *The Australian Society for Medical Research South Australian Division Annual Scientific Conference Proceedings*, Oral presentation.

**Bucco O**, Leifert WR, Aloia, AL, Burnard, SL, Barritt, GJ and McMurchie, EJ (2005) Designing novel cell-free drug screening platforms using captured G-protein coupled receptor 'transduosomes'. *BIO 2005 Innovation corridor poster session proceedings*. Poster presentation.

Aloia AL, Leifert WR, Glatz RV, **Bucco O**, Burnard SL and McMurchie EJ (2005) Construction of a surface immobilised G-protein coupled receptor biosensor. *Proceedings of the Australian Society for Biomaterials* p62.

Leifert WR, Aloia AL, **Bucco O** and McMurchie EJ (2005) G-protein coupled receptor signalling involves dissociation of G-protein subunits. *Proceedings of the Australian Society for Medical Research*.

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<sup>#</sup> Full paper

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## ABBREVIATIONS

$\beta_1$ AR-  $\beta$ -adrenergic receptor  
 $\alpha_{2A}$ AR-  $\alpha_{2A}$  adrenergic receptor  
[<sup>35</sup>S]GTP $\gamma$ S- radioactive isotope [<sup>35</sup>S] conjugated to GTP $\gamma$ S  
AAGR- average annual growth rate  
AC- adenylate cyclase  
AlF<sub>4</sub><sup>-</sup>- aluminium fluoride  
AMP- adenosine monophosphate  
Ang II- Angiotensin II  
AT<sub>1A</sub>- Angiotensin II type 1  
ATP- adenosine triphosphate  
Bp- base pairs  
BRET- bioluminescence resonance energy transfer  
BSA- bovine serum albumin  
cAMP- cyclic adenosine monophosphate  
CAMs- constitutively active receptor mutants  
CHN- CSIRO Division Human Nutrition \*  
CHO- Chinese hamster ovary cells  
CMC- critical micelle concentration  
CMHT- CSIRO Division of Molecular and Health Technologies \*  
CSIRO- Commonwealth Scientific and Industrial Research Organisation  
Da- daltons  
DMF- dimethylformide  
DMSO- dimethyl sulphoxide  
DNA- deoxyribose nucleic acid  
DTT- dithiothreitol  
*E.coli*- *Escherichia coli*  
EC<sub>50</sub>- effective concentration at which half the maximum effect is achieved  
FBS- foetal bovine serum  
FPR- formyl peptide receptor  
FRET- fluorescence energy transfer  
G $\alpha$ - G-protein alpha subunit  
GAP- GTPase activating protein  
GAPS- $\gamma$ -aminopropylsilane  
GDI- guanosine dissociation inhibitor  
GDP- guanosine diphosphate  
GEF- guanosine exchange factor  
GF/C- glass fibre filter mats  
GPCR- G-protein coupled receptor  
G-protein- heterotrimeric guanine nucleotide (guanosine) binding protein  
GTP- guanosine triphosphate  
GTP $\gamma$ S- guanosine 5-O-(3-thiotriphosphate)  
HPLC- high-performance liquid chromatography  
HTS- high throughput screening  
i.e.- in other words; that is to say that  
IC<sub>50</sub>- inhibitory concentration at 50% or also referred to as the molar concentration of an antagonist that reduces the response to an agonist by 50%.

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\* Formerly the CSIRO Division Health Sciences and Nutrition



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ICYP- [<sup>125</sup>I]-(-) Iodocyanopindolol  
IMAC-immobilised metal affinity chromatography  
IP<sub>3</sub>- inositol trisphosphate  
k<sub>assoc</sub>- rate of association  
k<sub>cat</sub>- overall reaction rate of catalysis  
K<sub>d</sub>- dissociation constant  
k<sub>dissoc</sub>- rate of dissociation  
k<sub>hydro</sub>- rate of hydrolysis  
MOI- multiplicity of infection  
Ni(NTA)- nickel-nitrilotriacetic acid  
NMR- nuclear magnetic resonance spectroscopy  
PEG- polyethylene glycol  
PEI- polythyleneimine  
PFM-plaque forming units  
PMSF- phenylmethylsulfonyl  
RAMPs- receptor activity modifying proteins  
RGS- regulator of G-protein signalling  
S/B- signal to background  
S/N- signal to noise  
SD- standard deviation  
SDS- PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis  
SEM- standard error of the mean  
*Sf9- Spodoptera frugiperda 9*  
SPA- scintillation proximity assay  
SPR- surface plasmon resonance  
TIFR- total internal fluorescence refraction  
UK-14304- synthetic adrenaline analogue