Immunochemical strategy for quantification of G-coupled olfactory receptor proteins on natural nanovesicles

Marta Sanmarti-Espinal, R Galve, Patrizia Iavicoli, Marie-Annick Persuy, Edith Pajot, M.P. Marco, Josep Samitier

► To cite this version:

Marta Sanmarti-Espinal, R Galve, Patrizia Iavicoli, Marie-Annick Persuy, Edith Pajot, et al.. Immunochemical strategy for quantification of G-coupled olfactory receptor proteins on natural nanovesicles. *Colloids and Surfaces B: Biointerfaces*, 2016, 139, pp.269-276. 10.1016/j.colsurfb.2015.11.062 . hal-01594487

HAL Id: hal-01594487

<https://hal.science/hal-01594487>

Submitted on 26 Sep 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Immunochemical strategy for quantification of G-coupled olfactory receptor proteins on natural nanovesicles

Marta Sanmartí-Espinal^{a,b,*}, Roger Galve^{c,d}, Patrizia Iavicoli^{a,1}, Marie-Annick Persuy^e, Edith Pajot-Augy^e, M.-Pilar Marco^{c,d}, Josep Samitier^{a,b,d,*}

^a IBEC—Institute for Bioengineering of Catalonia, Nanobioengineering Group, C/Baldiri Reixac, 15-21, 08028 Barcelona, Spain

^b Department of Electronics, University of Barcelona, C/Martí i Franquès 1, 08028 Barcelona, Spain

^c Nb4D—Nanobiotechnology for Diagnostics, IQAC-CSIC, C/Jordi Girona, 18-26, 08034 Barcelona, Spain

^d Centro de Investigación Biomédica en Red en Bioingeniería Biomateriales y Nanomedicina (CIBER-BBN), C/María de Luna 11, Edificio CEEI, 50018 Zaragoza, Spain

^e NBO, INRA, Université Paris-Saclay, 78350 Jouy-en-Josas, France

ARTICLE INFO

Article history:

Received 20 April 2015

Received in revised form 6 October 2015

Accepted 23 November 2015

Available online 11 December 2015

Dedicated to the memory of Francisco Sánchez Baeza.

Keywords:

G-protein-coupled receptors quantification
Competitive ELISA
Natural vesicles
Olfactory receptors
Transmembrane proteins
Bioelectronic nose

ABSTRACT

Cell membrane proteins are involved in a variety of biochemical pathways and therefore constitute important targets for therapy and development of new drugs. Bioanalytical platforms and binding assays using these membrane protein receptors for drug screening or diagnostic require the construction of well-characterized liposome and lipid bilayer arrays that act as support to prevent protein denaturation during biochip processing. Quantification of the protein receptors in the lipid membrane arrays is a key issue in order to produce reproducible and well-characterized chips. Herein, we report a novel immunochemical analytical approach for the quantification of membrane proteins (i.e., G-protein-coupled receptor, GPCR) in nanovesicles (NVs). The procedure allows direct determination of tagged receptors (i.e., c-myc tag) without any previous protein purification or extraction steps. The immunochemical method is based on a microplate ELISA format and quantifies this tag on proteins embedded in NVs with detectability in the picomolar range, using protein bioconjugates as reference standards. The applicability of the method is demonstrated through the quantification of the c-myc-olfactory receptor (OR, c-myc-OR1740) in the cell membrane NVs. The reported method opens the possibility to develop well-characterized drug-screening platforms based on G-coupled proteins embedded on membranes.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

G-protein-coupled receptors (GPCRs) are a large family of seven-transmembrane domain proteins involved in cell-response pathways to a variety of external signals and important ligands, such as hormones, neurotransmitters, and inflammatory mediators [1,2,3,4]. In fact, GPCRs are targeted by approximately 60% of all therapeutic drugs [5]. Therefore, technology platforms (i.e., bioassay and biosensor microarrays) for drug screening based on the use of these receptors would improve the efficiency of the

drug development pipeline at pre-clinical stages. Receptor binding assays developed for this purpose require building liposomes or bilayer arrays [6,7], that act as support for these transmembrane proteins to prevent denaturation during biochip processing and to ensure their biofunctionality [8–11]. Native liposomes, isolated directly from cell sources containing recombinant membrane proteins, retain the fluidity and lipid order of the original cell membrane, parameters that are relevant to maintain the functionality of the receptor [12,13]. Natural vesicles (NVs) produced from genetically engineered cells have been reported to be promising building blocks for sensing biodevices [14–19].

Deciphering the number of functional receptor molecules on a biochip or sensor array is a key issue in order to accomplish reliable and reproducible results. In addition to peptide tags combined with covalent labeling [20] or mutagenesis assays [21], GPCRs can be determined by methods such as single-molecule [22] or TIRF microscopy [23]. However, these approaches are not suitable for the development of well-characterized membrane-based protein

* Corresponding author at: IBEC—Institute for Bioengineering of Catalonia, Nanobioengineering Group, C/Baldiri Reixac, 15-21, 08028 Barcelona, Spain. Fax: +34 93 4037181.

E-mail addresses: msanmarti@ibecbarcelona.eu (M. Sanmartí-Espinal), jsamitier@ibecbarcelona.eu (J. Samitier).

¹ Current address: Joint Research Center, European Commission, Via Enrico Fermi 27491, 21027-Ispra, VA, Italy.

biosensor arrays. For this reason, we have focused on developing a straightforward method for detecting and quantifying tagged-membrane proteins or receptors such as c-myc-GPCRs in the form of lipidic NVs. The tag peptide can be fused to either the N- or the C-terminus of the protein of interest without changing the tertiary structure [24] or biological activity of the protein [25]. Although other techniques such as fluorescence polarization [26] have been used to quantify c-myc fused proteins, such approaches allow the quantification of only c-myc-protein concentration and not the number of c-myc-protein molecules per NV. To the best of our knowledge, this is the first report that accurately quantifies c-myc-tagged-GPCRs directly in the form of lipidic NVs.

2. Material and methods

2.1. Chemicals and immunochemicals

Biochemical reagents such as c-myc peptide (EQKLISEEDL) were purchased from Sigma Chemical Co. (St. Louis, MO). The commercial monoclonal anti-c-myc IgG1 9E10 antibody was supplied by Roche Diagnostics (Mannheim, Germany). *N*-succinimidyl 3-maleimidopropionate ($M(CH_2)_2CO$) and *N*-succinimidyl iodoacetate (CH_2CO) cross-linkers were synthesized in our laboratory as previously described [27,28]. Two modified c-myc peptides (peptide EQKLISEEDL-Cys, named C_1 , and peptide Cys-EQKLISEEDL, named C_2) were synthesized as C-terminal amides using standard Fmoc solid-phase protocols by the Platform of Peptide Synthesis by CIBER-BBN Biomedicine Scientific Services (<http://www.ciber-bbn.es>). The two peptides were characterized using mass spectrometry. Purities were >94%, as assessed by HPLC (data not shown). These peptides were used as haptens for the preparation of bioconjugates and as analytes for the competitive assays. The corresponding bioconjugates were characterized by MALDI-TOF-MS. Information on the suppliers of the other chemical and biochemical reagents used, is provided in the Electronic Supplementary Information (ESI).

2.2. Buffers and solutions

Phosphate buffer saline (PBS, 10 mM) solution was prepared as a 0.8% saline solution at pH 7.5. The PBST buffer contained PBS with 0.05% Tween 20 and the potassium dihydrogen phosphate/disodium hydrogen phosphate (PBT, 10 mM) buffer was prepared at pH 7.5 with 0.05% Tween 20. The substrate solution contained 0.01% TMB (3,3',5,5'-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer. TMB acts as a hydrogen donor for the reduction of hydrogen peroxide to water by horseradish peroxidase (HRP), producing a diimine with a blue color ($\lambda = 650$ nm). The addition of acid such as H_2SO_4 changes the colour to yellow ($\lambda = 450$ nm). More details about the general buffers/solutions are given in the ESI.

2.3. Equipment/Software

See the ESI.

2.4. Synthesis of peptide-bioconjugates

The two peptide haptens (C_1 , C_2) were covalently linked to keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), conalbumin (CONA), ovalbumin (OVA), horseradish peroxidase (HRP), and aminodextrane (AD) by means of *N*-succinimidyl 3-maleimidopropionate ($M(CH_2)_2CO$) or *N*-succinimidyl iodoacetate (CH_2CO) cross-linkers at different peptide:Lys (protein) molar ratios (for additional information on the complete procedure, see ESI) using procedures previously reported [27,29] (Table 1). Tables 1-S and 2-S (ESI) provide additional information regarding the

synthesized bioconjugates and the corresponding MALDI-TOF-MS recorded to obtain information on the number of attached peptide molecules. The bioconjugates were named aC_b -Y-X, where a is the true hapten density (δ) calculated by MALDI-TOF-MS, C_b the peptide (C_1 , C_2), Y the cross-linker ($M(CH_2)_2CO$ or CH_2CO), and X the macrobiomolecule used.

2.5. Monoclonal antibody production

The anti-c-myc monoclonal antibody (stock solution: 1.44 mg mL^{-1} , IgG mAb) was produced by Abyntek Biopharma (Bizkaia, Spain) in Balb/c mice, using the C_2 peptide conjugated to KLH with a maleimido derivative cross-linker as immunogen. The fused cells (spleen B cells from the selected mice with SP2\0 mouse myeloma cell line) were screened by competitive ELISA (see procedure below). For this purpose, we measured the binding of the supernatants to xC_2 - CH_2CO -CONA (where x corresponds to the bioconjugate synthesized at a ratio of 2:1:1, Tables Table 2 and 2-S) and 5C_1 - $M(CH_2)_2CO$ -CONA (0.2 μg mL^{-1} each) immobilized on the microtiter plates in the absence (zero concentration) or presence of the c-myc commercial peptide (ranging from 20 nM to 25 μM). The selected clones were subcloned again and subsequent screenings were performed in the same conditions. After these subsequent screenings, three cell clones were selected to produce the antibodies named Ab894D12_{d9d10}, Ab894D12_{f7d12}, and Ab894D12_{f7f9} (Tables 3-S and 4-S).

2.6. Preparation of the protein-receptor nanovesicle solutions

The human olfactory receptor c-myc-OR1740 (ORL520 in OrDB) and pJH2-somatostatin receptor subtype 2 (SSTR2), used as negative control, were expressed heterologously in *Saccharomyces cerevisiae* yeast cultures, as previously described [9]. The yeast cells were mechanically disrupted [19] and the cell content separated. The membrane fractions were obtained, divided in aliquots, and frozen at $-80^\circ C$ (~ 5 mg mL^{-1} , stock suspension). As a GPCR membrane receptor model, the fractions containing SSTR2 were used to evaluate the matrix effect of the immunochemical assay established for the quantification of c-myc-OR1740 integrated in the NVs. These NVs were produced and characterized as described in a recent publication by our group [30]. In brief, the characterization consisted in measuring: (i) total protein content (TPC) of NVs using the BCA Protein Assay; (ii) the average size of NVs through Dynamic Light Scattering and Cryo-EM; (iii) the concentration of NVs in the solutions (NV mL^{-1}) (Fig. 1-S) using Nanoparticle Tracking Analysis; and (iv) the zeta-potential of NVs in solution by using the Malvern Zetasizer instrument (Malvern Instruments, UK).

To perform the ELISA immunoassay, the stock suspensions of the membrane fractions were diluted to a TPC of 300 μg mL^{-1} in PBT and sonicated for 20 min in ice-cold water to homogenize the NV size. The solution was further diluted in PBT to a TPC of 60 μg mL^{-1} and passed through a sterile low protein-binding filter (Millipore, 13 mm diameter and 0.22 μm pore size). Finally, working solutions were obtained by adding PBT buffer until the desired concentration of NVs was reached. The SSTR2 blank matrix solution refers to PBT solutions of the NVs carrying the SSTR2 receptor without the c-myc peptide. The blank matrix was used at different concentrations (ranging from 10 to 50 μg mL^{-1} of TPC, or $1.47 \times 10^{10} - 7.33 \times 10^{10}$ NV mL^{-1}) [30] in the distinct experiments. The c-myc-OR1740 NV solutions were consistently analyzed at the same TPC concentrations as the reference SSTR2 blank matrix.

For the Western blot assays, 1 μL of the stock membrane suspension was loaded on each well of the gel (5 μg per well), and the assays were performed following the procedure described by Minic et al. [9] (see ESI for more details).

Table 1

Relation of hapten densities achieved for the peptide-macrobimolecule bioconjugates and the cross-linker used for the bioconjugation reaction.

Heterobifunctional Cross-linkers	M(CH ₂) ₂ CO ^a		CH ₂ CO ^a	
Peptide	Bioconjugate	δ^b	Bioconjugate	δ^b
C ₁ : EQKLISEEDLCys	C ₁ -M(CH ₂) ₂ CO-BSA	1/3 ^c	C ₁ -CH ₂ CO-BSA	1
	C ₁ -M(CH ₂) ₂ CO-OVA	–	C ₁ -CH ₂ CO-OVA	1
	C ₁ -M(CH ₂) ₂ CO-CONA	4/5/6 ^c		
	C ₁ -M(CH ₂) ₂ CO-HRP	1		
	C ₁ -M(CH ₂) ₂ CO-AD	–		
C ₂ : CysEQKLISEEDL			C ₂ -CH ₂ CO-BSA	6/8 ^c
			C ₂ -CH ₂ CO-OVA	3/5 ^c
			C ₂ -CH ₂ CO-CONA	–
			C ₂ -CH ₂ CO-HRP	1
			C ₂ -CH ₂ CO-AD	–

–Bioconjugate that could not be characterized with the MALDI-TOF-MS conditions used.

^a M(CH₂)₂CO or CH₂CO refers to the spacers resulting from the bioconjugation reaction using N-succinimidyl 3-maleimidopropionate or N-succinimidyl iodoacetate as cross-linkers, respectively.^b number of peptide residues bound to the protein according to MALDI-TOF-MS analysis. Additional information on the preparation and characterization of these conjugates can be found in the ESI.^c bioconjugate synthesized at different molecular concentration (Peptide:Crosslinker:Lys).**Table 2**

Immunoassay features achieved for c-myc and the protein bioconjugates.

mAb	Ab9E10	Ab894D12 ₁₇₁₉							
Buffer	PBST	PBST	PBT						Blank Matrix ^a
CA ^b	⁵ C ₁ -CONA ^b	⁵ C ₁ -CONA ^c	⁵ C ₁ -CONA ^d	³ C ₂ -CH ₂ CO-OVA ^e	³ C ₂ -CH ₂ CO-OVA ^e	¹ C ₁ -HRP ^g	³ C ₁ -BSA ^g	⁴ C ₁ -CONA ^g	¹ C ₁ -HRP ^g
Target analyte ^h	c-myc ^f	c-myc ^f	c-myc ^f	c-myc ^g	C ₂ ^g				
A _{max}	1.40 ± 0.02	0.75 ± 0.01	1.15 ± 0.01	0.89 ± 0.01	0.87 ± 0.01	0.87 ± 0.01	0.90 ± 0.01	0.86 ± 0.01	0.90 ± 0.01
A _{min}	0.04 ± 0.04	0.05 ± 0.01	0.07 ± 0.01	0.11 ± 0.02	0.08 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.14 ± 0.01
IC ₅₀ , nM	1318 ± 0.05	8.02 ± 0.05	7.84 ± 0.03	19.0 ± 0.05	2.72 ± 0.06	0.99 ± 0.04	0.08 ± 0.02	0.07 ± 0.03	0.39 ± 0.04
Slope	–1.07 ± 0.12	–1.03 ± 0.11	–0.99 ± 0.07	–1.01 ± 0.09	–0.80 ± 0.07	–0.85 ± 0.06	–0.90 ± 0.03	–1.15 ± 0.03	–0.96 ± 0.07
R ²	0.99 ± 0.05	0.99 ± 0.03	1.00 ± 0.03	0.99 ± 0.03	0.99 ± 0.03	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.02	0.99 ± 0.03

^a SSTR2-NV solution (TPC = 35 µg mL^{–1}).^b CA 0.062 (µg mL^{–1}).^c CA 0.031 (µg mL^{–1}).^d CA 0.016 (µg mL^{–1}).^e CA 0.010 (µg mL^{–1}).^f mAb dilution used was 1/4000.^g mAb dilution used was 1/250000.^h Unless otherwise indicated the cross-linker of the bioconjugates is M(CH₂)₂CO.**Table 3**Quantification of ORs per NV using the different protein bioconjugates as standard references^a.

c-myc-OR1740		OR NV ^{–1,b}			ORs NV ^{–1,c}
[TPC], µg mL ^{–1}	[Vesicles] NV mL ^{–1}	³ C ₁ -M(CH ₂) ₂ CO-BSA	⁴ C ₁ -M(CH ₂) ₂ CO-CONA	¹ C ₁ -M(CH ₂) ₂ CO-HRP	x ₀ x _r
25	7.0 × 10 ¹⁰	1.04 ± 0.17	1.80 ± 0.34	–	1.42 ± 0.54
30	8.4 × 10 ¹⁰	2.04 ± 0.10	4.69 ± 0.22	–	3.37 ± 1.88
35	9.8 × 10 ¹⁰	1.32 ± 0.01	2.86 ± 0.02	–	2.09 ± 1.08
		3.79 ± 0.37	4.52 ± 0.37	5.53 ± 0.56	4.61 ± 0.87
45	12.6 × 10 ¹⁰	2.04 ± 0.02	3.81 ± 0.04	–	2.92 ± 1.25
		1.46 ± 0.03	3.01 ± 0.05	2.93 ± 0.05	2.47 ± 0.87

–Any concentration of OR per nanovesicle was obtained after interpolation of the immunochemical response that was out of the linear range (20–80% of the maximum absorbance) of the corresponding standard curve of the bioconjugate.

^a According to the linear correlation between the Total Protein Concentration (TPC, µg mL^{–1}) and the nanovesicle concentration (NV mL^{–1}) a ratio of 3.6 × 10 pg protein/NV is estimated (see Supporting information). Results were obtained on different days.^b Concentration of OR per nanovesicle resulting from interpolating the immunochemical response of each solution onto the corresponding standard curve of each bioconjugate. Calculations were made, as previously described (see experimental section and Table 8-S) considering the δ of each bioconjugate.^c ORs concentration per nanovesicle calculated as average of the measurements using the different bioconjugates as standards for each TPC (x₀) and taking into consideration all measurements made at different TPC (x_r).

2.7. Competitive ELISAs

Appropriate concentrations of the immunoreagents were determined through checkerboard titration experiments. For this purpose, we measured the binding of serial dilutions of the antibodies (ranging from 9.76 × 10^{–3} – 10 µg mL^{–1} and undiluted in PBST, using 100 µL per well) to the microtiter plates coated with different concentrations of the bioconjugates (7.81 × 10^{–3} – 1 µg mL^{–1} and undiluted in coating buffer, using 100 µL per well), as previously described [31]. All washing steps consisted of four cycles of

filling the wells with PBST (300 µL) and immediately aspirating the solutions by vacuum with an automated microplate washer. Whenever NV samples or standards were measured, the microplates were agitated at 600 rpm during the competitive step.

2.7.1. Nanovesicle ELISA procedure

Microtiter plates were coated with the antigen (³C₂-CH₂CO-OVA, 0.01 µg mL^{–1} in coating buffer, 100 µL per well) for 4 h at room temperature covered with adhesive sealers. Next, the plates were washed and solutions of the c-myc-OR1740 NV samples and/or the

bioconjugate standards ($^4\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-CONA}$, $^3\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-BSA}$, and $^1\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-HRP}$, 1000–0.008 nM in PBT or in the SSTR2 NV matrix) were added (50 μL per well), followed by the monoclonal antibody (Ab894D12₁₇₁₉, 1/250000 in PBT or in the SSTR2 NV matrix, 50 μL per well). The mixture was incubated for 30 min at room temperature and the plates were then washed again with PBST. Subsequently, a solution of anti mouse-IgG-HRP (1/6000 in PBST, 100 μL per well) was added and the plates were incubated for an additional 30 min and washed before adding the substrate solution (0.01% TMB and 0.004% H_2O_2 in citrate buffer, 100 μL per well). After 30 min at room temperature, the enzymatic reaction was stopped by adding 4N H_2SO_4 (50 μL per well) and absorbance was measured at 450 nm.

The standard curves were fitted to a four-parameter logistic equation according to the following formula: $Y = (A - B) / [1 + (x/C)^D] + B$, where A stands for the maximal absorbance, B is the minimum absorbance, C represents the concentration producing 50% of the difference between the maximal and the minimal absorbance (also IC50), and D is the slope at the inflection point of the sigmoid curve. Unless stated otherwise, all data shown here correspond to the average of the three well replicates.

2.8. Quantification of olfactory receptors through the c-myc tag

The sample containing NVs carrying an unknown number of c-myc-tagged OR1740 was diluted at several TPC concentrations in PBT and measured using the ELISA procedure described above. In these experiments, the standard curves were built in SSTR2 blank matrices containing the same TPC as in the samples. The three bioconjugates ($^4\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-CONA}$, $^3\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-BSA}$, and $^1\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-HRP}$) were used as standard references taking into consideration the number of peptides linked to the macro-biomolecule, as calculated by MALDI-TOF-MS. By interpolating the immunochemical response of the NV solutions onto the calibration curves built with these standards, we were able to calculate the number of c-myc molecules in the solutions, and thus the number of c-myc-OR1740 receptors using the Avogadro number (Eqs. (1) and (2)). Taking into consideration that each OR has only one c-myc tag and the concentration of NVs in solution, it was possible to obtain an estimate for the average number of c-myc-OR1740 among our NVs (Eq. (3)). Note that both c-myc-olfactory receptor orientations are possible after NV preparation but only those c-myc tags externally oriented in the NV were quantified.

At each NV concentration, the absorbance average was interpolated onto each of the three standard curves prepared with the c-myc-bioconjugates models. Considering the corresponding c-myc:bioconjugate molar ratio calculated by MALDI-TOF-MS (δ_{MALDI}), we can directly correlate this concentration with the concentration of c-myc tag (Eq. (1)).

$$\begin{aligned} \delta_{\text{MALDI}} \times \text{C}_1\text{-bioconjugate (nmolL}^{-1}) &\equiv \text{c-myc (nmolL}^{-1}) \\ &\equiv \text{olfactoryreceptor (nmolL}^{-1}) \end{aligned} \quad (1)$$

Since each olfactory receptor is fused to one single c-myc tag, it is possible to obtain the molarity of the olfactory receptors in the sample solution (Eq. (1)) and calculate the number of olfactory receptors per unit volume (Eq. (2)) considering the c-myc concentration average from the three model bioconjugates.

$$\begin{aligned} \text{olfactoryreceptor (nmolL}^{-1}) &\times \frac{1 \text{ mol}}{10^9 \text{ nmol}} \times \frac{6.022^{23} \text{ molecules}}{1 \text{ mol}} \\ &= \text{OR (moleculesL}^{-1}) \end{aligned} \quad (2)$$

Fig. 1. Schematic of the natural lipidic nanovesicles carrying the c-myc-OR1740 expressed by genetic engineering. Zoom of the quantified complex: OR1740 as a seven-transmembrane protein with the c-myc peptide (10 amino acids) fused to its N-terminus.

The number of olfactory receptors per NV can be quantified from the correlation between the total amount of protein vs the number of NVs and olfactory receptors per mL (Fig. 1-S, Eq. (3)).

$$n^{\circ} \text{ORNV}^{-1} = \frac{(\delta_{\text{MALDI}} \cdot [\text{Bioconjugate}])}{\text{TPC}} \cdot K_m \quad (3)$$

where K_m is a constant rate of $6.022 \times 10^{11}/\text{m}$ and m is $2.79 \times 10^9 \text{ NV } \mu\text{g}^{-1}$ (Fig. 1-S) for c-myc-OR1740 NVs.

3. Results and discussion

The c-myc sequence (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) is one of the most used peptide tags in protein expression. Here, we used c-myc-OR1740 as a model to study the GPCR family. The c-myc was attached through its -COOH terminal amino acid to the -NH₂ terminal amino acid of the protein, leaving the NH₂ terminus of the c-myc tag exposed to the external media on the prepared OR-NVs (Fig. 1).

The determination of the average number of protein receptors per NV has always been challenging. In order to ensure reproducibility and potential biofunctionality of each NV batch, the total protein concentration (TPC) – rather than just the receptor – is typically used to estimate the amount of receptor in these preparations. Moreover, the expression of tagged recombinant proteins is a common strategy for surface immobilization (i.e., biosensing platforms [14,15]). However, the lack of information on the number of tag-GPCR molecules embedded in NVs is an important drawback, which is why we developed an immunochemical analytical method to quantify the receptor in NV cell membranes.

3.1. Bioconjugates, synthesis and characterization

Antibodies against c-myc were selected using two c-myc-related hapten peptides as coating antigens (C_1 and C_2 , see Table 1 for peptide sequences), bearing an additional Cys residue for conjugation at the carboxy and the amino terminus, respectively (see below). The thiol group of the Cys was used as nucleophile for coupling the haptens to the crosslinkers. These peptides were then coupled to the macromolecules (KLH, BSA, CONA, OVA, HRP, and AD) [32] at different molar ratios (Tables 1, 1-S and 2-S). The KLH conjugates were used for antibody production, while the rest were used as immunoreagents for the assay development.

Two different crosslinkers were selected in order to avoid any recognition of the spacer arm from the obtained antibodies. The albumins BSA, CONA, and OVA were selected because they are common proteins and are widely used to prepare bioconjugates. HRP was chosen because of the low number of Lys residues available, allowing bioconjugation ratios close to the ideal (hapten:protein,

1:1). Finally, AD was selected because of its hydrophilic character, in order to minimize possible non-specific absorptions onto the lipidic NVs, and to increase the solubility of the bioconjugates in the NV matrices [32]. All the bioconjugates were characterized by MALDI-TOF-MS to determine the c-myc:protein ratio (Table 2-S).

We prepared a large number of bioconjugates because we needed to obtain a good model that could mimic the behaviour of the c-myc-OR1740 NVs in the competitive assays. The strategy was based on the idea that bioconjugates should have affinity constants for the antibody, similar to the c-myc OR [33].

3.2. Antibody, production and characterization

Balb/c mice were immunized with the KLH bioconjugates and splenocytes were fused to B cells to generate hybridomas. We performed screening assays to select the best B cell clones that produced high affinity monoclonal antibodies by measuring the binding of the antibody fraction in supernatants to the C₁ and C₂ bioconjugates immobilized on the microtiter plates. The objective was to select clones that are able to recognize most of the c-myc peptide structure in order to ensure satisfactory performance in the developed immunochemical assay (Tables 3-S and 4-S). C₁ bioconjugate perfectly mimics the orientation of c-myc on the c-myc-OR1740 receptor allowing the NH₂ terminus of the c-myc peptide to be exposed to the external media in a similar way as the c-myc-olfactory receptor. C₂ bioconjugates could also assist with the selection of clones, showing an equally high affinity against amino acids in the center of the c-myc tag sequence or the area close to the OR. The screening assays were performed in the presence (competitive conditions) and absence (non-competitive conditions) of the target analyte (c-myc), in order to mimic the conditions envisaged for the immunochemical assay (Fig. 2). High antibody titers were obtained from mice immunized with the C₂-KLH bioconjugate, isolating three antibody clones with acceptable features in terms of signal and detectability. In PBST, Ab894D12_{f7f9} provided the best immunoassay features using ⁵C₁-M(CH₂)₂CO-CONA as competitor (coating antigen) in an indirect ELISA format (Table 2, Fig. 2A.I). Fig. 3A shows the calibration curves obtained in this immunochemical system using two antibody sources (Ab894D12_{f7f9} and Ab9E10). Calibration curves for ligand binding assays [34] such as a competitive ELISA (Fig. 3) are generally characterized by a non-linear relationship between the mean response and the analyte concentration, giving an absorbance response that decreases as the concentration of competitor analyte increases. The assay features obtained after fitting the curve to a four-parameter equation are shown in Table 2.

The detectability using Ab894D12_{f7f9} was two orders of magnitude greater than that reached with the Ab9E10 antibody obtained from commercial sources (IC₅₀ values varied from ~1000 nM to ~10 nM in PBST buffer using Ab9E10 or Ab894D12_{f7f9}, respectively). This difference was attributed to the particular immunizing hapten used. While the Ab894D12_{f7f9} antibody was produced against the c-myc sequence (Cys-EQKLISEEDL), the commercial antibody 9E10 had a longer peptide sequence (AEEQKLISEEDLLRKRREQLKHLEQLRNSCA) conjugated through the Cys residue (C) to KLH as immunizing hapten. Although Ab9E10 recognizes the EQKLISEEDL sequence from the human c-myc protein [35] in solution or when linked to the carrier, this peptide could have folded into the tertiary structure of the carrier, thus diminishing the capacity of these antibodies to strongly bind the c-myc peptide. In independent experiments, our group used other commercial anti-c-myc antibodies with the c-myc-olfactory receptor nanovesicles which yielded a lower affinity than Ab9E10 (not shown).

In order to improve detectability, we evaluated the effect of several physicochemical factors (such as pH, ionic strength, pres-

ence of Tween 20, as well as incubation and competition times) on the ELISA immunochemical assay with the result that only the ionic strength produced a significant effect (see ESI). Fig. 3A demonstrates how the standard curves built in PBT exhibited a notably higher signal. We therefore presumed that we had produced a highly sensitive and selective monoclonal antibody capable of detecting the c-myc tag in the nanomolar range. The capacity of Ab894D12_{f7f9} to recognize the c-myc-OR1740 embedded in the NV results was further confirmed by Western blot assays (Fig. 4).

3.3. Development of an ELISA for c-myc-tagged transmembrane proteins

The protein receptor (OR1740) has been expressed in yeast plasma membranes [9], a system considered suitable to achieve high protein expression [36,37]. NVs were prepared from these cells as described in Section 2 and characterized in respect to their average size, concentration (NV mL⁻¹), charge, and TPC. In this regard, we have previously demonstrated [30] that there is a correlation between TPC and NV concentration. We estimated that the NV batch used in this study had a protein content of 3.6×10^{-4} pg NV⁻¹ (Fig. 1-S).

As the NVs (diameter ≈ 100 nm) containing the c-myc-OR1740 were formed from natural membranes, they contain a high content of phospholipids that comprise the core structure of cell membranes. Our main concern was therefore the potential non-specific interactions of the lipophilic NVs with the immunoreagents or the labware used in the assays. In order to assess these potentially undesirable effects, we built calibration curves on solutions of blank matrices and compared the immunochemical response to the curve prepared using buffer. These blank matrices were solutions of different concentrations of NVs (different TPC) prepared under the same conditions, but carrying another transmembrane receptor (SSTR2) without the c-myc tag. The response of the calibration curves built from these blank matrices ($3\text{--}60 \mu\text{g mL}^{-1}$ TPC or $4.40 \times 10^9\text{--}8.80 \times 10^{10}$ NV mL⁻¹) did not differ significantly from the curve prepared using buffer (Figs. 3B and 2-S). Only solutions with a high TPC concentration ($60 \mu\text{g mL}^{-1}$) yielded a slight decrease of the maximum absorbance leaving the detectability unaffected. This effect could be eliminated by simply diluting the solution 1:2 (to $20 \mu\text{g mL}^{-1}$) with PBT. As a result, the developed microplate-ELISA performed well on such media, despite the lipidic nature of the membrane components. The analytes used in our experiments to determine the matrix effect of the NVs were ³C₁-M(CH₂)₂CO-BSA and ⁴C₁-M(CH₂)₂CO-CONA (Fig. 2-S). Both bioconjugates were selected because they mimic the orientation of the c-myc peptide on the surfaces of the NVs.

The identification of a suitable reference for quantifying the c-myc-ORs on the NVs remains an important challenge. Since this tagged receptor is stable only when incorporated on membranes, a well-characterized NV with a defined number of OR molecules would be the ideal standard. The lack of analytical tools to characterize the NV from this point of view has made this approach unfeasible until now. In this study, we proposed the use of well-characterized c-myc-bioconjugates as standards rather than the c-myc peptide alone, since the former is better at mimicking c-myc-OR1740. By using ³C₁-Y-X bioconjugates, in which the c-myc was coupled through the N-terminal amino acid of the protein, the c-myc epitopes will be exposed for antibody recognition in a similar manner as the c-myc-OR in the NVs (Fig. 1).

We assessed the immunochemical response of three well-characterized bioconjugates with the lowest immunochemical assay detectability, IC₅₀, (⁴C₁-M(CH₂)₂CO-CONA, ³C₁-M(CH₂)₂CO-BSA, and ¹C₁-M(CH₂)₂CO-HRP, Table 5-S) including c-myc-OR in the NVs (Fig. 2A.II and A.III). For this purpose, calibration curves of bioconjugates were prepared in the blank matrix and results were

Fig. 2. Schematic representation of the indirect competitive ELISA experiments. (A.I.) Immunoassay scheme using the c-myc peptide as analyte to evaluate two anti-c-myc monoclonal antibodies (mAbs) (Ab9E10 and Ab894D12₇₇₁₉) and the absence of salts in the buffer. (A.II.) Immunoassay scheme using c-myc bioconjugates ($^3\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-BSA}$, $^4\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-CONA}$, or $^1\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-HRP}$) to perform the standard calibration curves in SSTR2-nanovesicles solution. (A.III.) Immunoassay scheme of our model for GPCR (c-myc-OR1740) quantification in the nanovesicle (NV) sample. (B) Experimental set-up performed on a 96 ELISA well-plate.

Fig. 3. (A) Calibration curves of the competitive immunoassays using the c-myc peptide as analyte and two anti-c-myc monoclonal antibodies, Ab9E10 and Ab894D12₇₇₁₉. The absence of salts in the buffer (phosphate buffer with 0.05% Tween-20, PBT (purple curve) instead of a phosphate buffer saline with 0.05% Tween-20, PBST (magenta curve)) resulted in a higher maximum signal of the assay. (B) Matrix effect produced by a solution of natural yeast nanovesicles. Standard curves using $^3\text{C}_1\text{-M}(\text{CH}_2)_2\text{CO-BSA}$ as analyte in PBT and in blank matrix buffer (SSTR2) at a range of concentrations (3–60 $\mu\text{g mL}^{-1}$ TPC or 4.40×10^9 – 8.80×10^{10} NV mL^{-1}). For the immunoassay features see ESI. (C) Calibration curves of the c-myc-bioconjugates chosen as candidates for the quantification model in the blank matrix buffer (SSTR2-NV, TPC = 35 $\mu\text{g mL}^{-1}$). Each curve was built using three-well replicates. The parameters shown in Table 2 were extracted from the four-parameter equation used to fit the standard curves.

analyzed using the developed microplate ELISA. In these experiments, $^3\text{C}_2\text{-CH}_2\text{CO-bioconjugates}$ were used as coating antigens since they provided greater detectability (Table 4-S) due to the greatest heterogeneity in respect to the immunogen (different cross-linker). The detectability achieved for the c-myc-bioconjugates was in the low nM range ($\text{IC}_{50}=0.083\text{--}0.386\text{ nM}$) for all cases (Figs. 3C and 3-S, Table 2). As expected, the immunochemical response was different, depending on the protein used and on the hapten density of each bioconjugate. Thus, the HRP bioconjugate with only one hapten attached, provided an indication of the lower detectability, while no significant differences were observed between the BSA and CONA bioconjugates. Controls using the maleimido-derivatized proteins as analytes demonstrated that

the response was only due to the c-myc peptide. There was no interference from the cross-linker that had also been used for the preparation of the immunogen (Fig. 4-S) since no inhibition curves had been obtained.

3.4. Olfactory receptor quantification

Solutions with different concentrations of c-myc-OR1740-NVs (in PBT) were measured using standard curves prepared with the three peptide-protein bioconjugates in the blank matrix mentioned above. First, as proof-of-concept, we tested the viability of the approach by quantifying the c-myc hapten load of c-myc protein bioconjugates (see ESI and Table 8-S). Only those NV solutions

Fig. 4. Image of the Western blot results showing the capacity of different concentrations (C) of anti-c-myc antibodies Ab9E10 and Ab894D12₁₇₁₉ to detect c-myc-OR1740. Nanovesicles carrying SSTR2 receptors were used as negative control (–). Nanovesicles carrying c-myc-OR1740 were used as evaluation sample (+). The monomer olfactory receptor molecular weight is ~35 kDa [38].

(TPC = 25–45 $\mu\text{g mL}^{-1}$) providing an immunochemical response within the lineal range of the assay (between 20 and 80% of the maximum absorbance on each of the three bioconjugate calibration curves) were used for quantization of the sample. The resulting immunochemical response (Table 3) was, as expected, very similar for the CONA and BSA bioconjugates and higher for the HRP bioconjugate, since the detectability provided by the latter is lower (a particular absorbance value is given by higher concentration values than for the other bioconjugates). By deconvoluting the concentration values found on the three calibration curves and taking into consideration the NV concentration (as described in Section 2 and Fig. 1–S), we found that our preparations contained approximately (2.8 ± 1.1) c-myc-OR1740 per NV.

Furthermore, the ORs quantification in one nanovesicle presented above has been compared with the quantification of solubilized ORs by the protein purification technique, a standard well-known experimental procedure based on solubilizing all the proteins from the yeast lipid membranes with a detergent and purifying the c-myc-ORs onto an affinity column with an anti-c-myc antibody (see ESI).

Subsequent absorbance quantification of the eluted purified receptors gives (0.0030 ± 0.0005) g c-myc-OR1740/g total proteins. Considering the molecular weight of the receptor (~35 kDa), there are 5.2×10^{16} OR/g total protein.

We can estimate the coherence of the values obtained from an easy theoretical quantification. The membrane volume necessary to form a 100 nm diameter-NV with a width of around 7.5 nm [39] is around $202,043 \text{ nm}^3$; the *S. cerevisiae* membrane protein density reported in the literature [40] is $1.15\text{--}1.17 \text{ g cm}^{-3}$ and its membrane cell composition (total protein composition) [41] is 49.3 g TP in 100 g of membrane; and finally, the recombinant GPCR expression level in this yeast host system is 0.003 gr OR for gr of total protein content. So, the theoretical estimated quantification of OR per NV expected would be around 6 (Eq. (4)).

$$202043 \text{ nm}^3 \text{ NV}^{-1} \times \frac{1 \text{ cm}^3}{1 \cdot 10^{21} \text{ nm}^3} \times \frac{1.16 \text{ g membrane}}{\text{cm}^3} \times \frac{49.3 \text{ g TP}}{100 \text{ g membrane}} \times \frac{5.2 \times 10^{16} \text{ OR}}{1 \text{ g TP}} \approx 6 \text{ OR NV}^{-1} \quad (4)$$

Thus, the quantification value obtained by the ELISA method, (2.8 ± 1.1) c-myc-OR1740 per NV, can be explain assuming that 50% of the ORs expressed on the NVs were oriented with the N-terminus (the c-myc tag) outside the membrane due to the nanovesicles fabrication process.

At this point, and to our knowledge, we can confirm that the quantification of receptors per NV using the ELISA method, as demonstrated in this study, is the best approach available today for the characterization of these preparations in a more accurate manner.

4. Conclusions

The novel immunochemical method described here has the capacity to quantify the number of olfactory receptors per NV in preparations of c-myc-transmembrane proteins embedded in such vesicles. We demonstrated the feasibility of this approach by using chemically prepared c-myc peptide-protein bioconjugates. The hapten density estimated from the immunochemical response of these bioconjugates using ELISA was very similar to that obtained by MALDI-TOF-MS. We used ELISA to measure NVs prepared from yeast expressing the human c-myc-OR1740 (c-myc-GPCR) which yielded a value of (2.8 ± 1.1) OR molecules per NV for the number of olfactory receptors embedded in the NVs. The possibility to obtain this parameter will allow for a much more accurate characterization of these preparations and highlight the expected biofunctionality. These findings are highly relevant for the continuing development of drug-screening platforms that use these bioreceptors as target biomolecules. The immunochemical method developed here, represents an innovative approach to determine the expression of membrane protein receptors in their natural environment without the need to isolate and purify them. This novel technique could also be applied in multiple fields, since the analysis of c-myc-tagged molecules, especially membrane proteins, continues to be a challenge in biology and biomedicine.

Acknowledgments

This work is supported by the “Nanoscience, nanotechnologies, materials and new production technologies” programme under the project BOND (228685-2) funded by the 7th Research Framework Programme of the European Union and by the FIS Project PI10/01171 funded by the *Instituto de Salud Carlos III* (Ministerio de Ciencia e Innovación)(Spain) and the Botín Foundation. MS was awarded a predoctoral fellowship by the *Instituto de Salud Carlos III*. The Nb4D group and Nanobioengineering SIC-BIO group are recognized by the *Generalitat de Catalunya* (Government of Catalonia) as consolidated Grups de Recerca and have support from the *Departaments d'Universitats, Recerca i Societat de la Informació of the Generalitat de Catalunya* (expedient 2009 SGR 1343 and 2009 SGR 505). CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008–2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions, and financed by the *Instituto de Salud Carlos III*, with assistance from the European Regional Development Fund. The authors also thank José Amable Bernabé (ICMAB/CSIC/CIBER BBN) for his help in Nanosight measurements and Dr. Karine Badonnel (INRA, Neurobiologie de l'Olfaction et Modélisation en Imagerie) for her help in olfactory receptors solubilization and purification experiments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2015.11.062>.

References

- [1] A.E. Brady, L.E. Limbird, *Cell Signal.* 14 (4) (2002) 297.
- [2] T. Schöneberg, A. Schulz, H. Biebertmann, T. Hermsdorf, H. Römpler, K. Sangkhul, *Pharmacol. Ther.* 104 (3) (2004) 173.
- [3] F.-M. Wurm, *Nat. Biotechnol.* 22 (11) (2004) 1393.
- [4] P.A. Insel, C.-M. Tang, I. Hahntow, M.C. Michel, *Biochim. Biophys. Acta -Biomembranes* 1768 (4) (2007) 994.
- [5] J. González-Maeso, *Nat. Neurosci.* 13 (5) (2010) 524.
- [6] Z.-K. Cui, M. Lafleur, *Colloids Surf. B* 114 (2014) 177.
- [7] G.H. Zana, N.-J. Choa, *Colloids Surf. B* 121 (2014) 340.
- [8] M. Bally, K. Bailey, K. Sugihara, D. Gieshaber, B. Vörös, J. Städler, *Small* 22 (6) (2010) 2481.
- [9] J. Minic, M.-A. Persuy, E. Godel, J. Aioun, I. Connerton, R. Salesse, E. Pajot-Augy, *FEBS J.* 272 (2) (2005) 524.
- [10] A. Bieri, O.P. Ernst, S. Heyse, H. Hofmann, K.P. Vogel, *Nat. Biotechnol.* 17 (11) (1999) 1105.
- [11] N.J. Wittenberg, H. Im, T.W. Johnson, X. Xu, A.E. Warrington, M. Rodriguez, S.H. Oh, *ACS Nano* 5 (9) (2011) 7555.
- [12] S. Mukherjee, F.R. Maxfield, *Annu. Rev. Cell Dev. Biol.* 20 (2004) 839.
- [13] K. Simons, W.L.C. Vaz, *Annu. Rev. Biophys. Biomol. Struct.* 33 (2004) 269.
- [14] J. Vidic, M. Pla-Roca, J. Grosclaude, M.-A. Persuy, R. Monnerie, D. Caballero, A. Errachid, Y. Hou, N. Jaffrezic-Renault, R. Salesse, E. Pajot-Augy, J. Samitier, *Anal. Chem.* 79 (2007) 3280.
- [15] J. Minic-Vidic, J. Grosclaude, M.-A. Persuy, J. Aioun, E. Salesse, R. Pajot-Augy, *Lab Chip* 6 (2006) 1026.
- [16] I. Casuso, M. Pla-Roca, G. Gomila, J. Samitier, J. Minic, M.A. Persuy, E. Salesse, R. Pajot-Augy, *Mater. Sci. Eng. C* 28 (2008) 686.
- [17] Y. Hou, N. Jaffrezic-Renault, C. Martelet, A. Zhang, J. Minic-Vidic, T. Gorjankina, M.-A. Persuy, E. Pajot-Augy, R. Salesse, V. Akimov, L. Reggiani, C. Pennetta, E. Alfinito, O. Ruiz, G. Gomila, A. Samitier, J. Errachid, *Biosens. Bioelectron.* 22 (2007) 1550.
- [18] J. Park, J.H. Lim, H.J. Jin, S. Namgung, S.H. Lee, T.H. Park, S. Hong, *Analyst* 137 (14) (2012) 3249.
- [19] J. Minic, J. Grosclaude, J. Aioun, M.-A. Persuy, T. Gorjankina, R. Salesse, E. Pajot-Augy, Y. Hou, S. Helali, N. Jaffrezic-Renault, F. Bessueille, A. Errachid, G. Gomila, J. Ruiz, O. Samitier, *Biochim. Biophys. Acta* 1724 (2005) 324.
- [20] H. Nonaka, S. Fujishima, S. Uchinomiya, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* 132 (27) (2010) 9301.
- [21] A. Dodevski, I. Plückthun, *J. Mol. Biol.* 408 (2011) 599.
- [22] A. Sergé, S. de Keijzer, F. Van Hemert, M.R. Hickman, D. Hereld, H.P. Spaink, T. Schmidt, B.E. Snaar-Jagalska, *Integr. Biol.* 3 (6) (2011) 675.
- [23] S.A. Mutch, P. Kensel-Hammes, J.C. Gadd, B.S. Fujimoto, R.W. Allen, P.G. Schiro, R.M. Lorenz, C.L. Kuyper, J.S. Kuo, S.M. Bajjalieh, D.T. Chiu, *J. Neurosci.* 31 (4) (2011) 1461.
- [24] D.-J. Manstein, H.-P. Schuster, P. Morandini, D.-M. Hunt, *Gene* 162 (1995) 129.
- [25] K. Terpe, *Appl. Microbiol. Biotechnol.* 60 (2003) 623.
- [26] S. Sun, L.-H. Nguyen, O.-H. Ross, R. Hollis, G.-F. Wynn, *Anal. Biochem.* 307 (2002) 287.
- [27] M.-C. Estévez, R. Galve, F. Sánchez-Baeza, M.-P. Marco, *Chem. Eur. J.* 14 (2008) 1906.
- [28] O. Nielsen, O. Buchardt, *Synthesis* 10 (1991) 819.
- [29] B.G. Pinacho, F. Sánchez-Baeza, M.-P. Marco, *Anal. Chem.* 84 (2012) 4527.
- [30] A. Calo, M. Sanmartí-Espinal, P. Iavicoli, M.-A. Persuy, E. Pajot-Augy, G. Gomila, J. Samitier, *Soft Matter* 8 (46) (2012) 11632.
- [31] M.C. Estévez, M. Kreuzer, F. Sánchez-Baeza, M.-P. Marco, *Environ. Sci. Technol.* 40 (2) (2006) 559.
- [32] R. Galve, F. Sanchez-Baeza, F. Camps, M.-P. Marco, *Anal. Chim. Acta* 452 (2002) 191.
- [33] H. Holthues, U. Pfeifer-Fukumura, I. Sound, W. Baumann, J. Immunol. Methods 304 (2005) 68.
- [34] J.W.A. Findlay, R.F. Dillard, *AAPS J.* 9 (2007) E269.
- [35] G.I. Evan, J.K. Lewis, J.M. Ramsay, G. Bishop, *Mol. Cell. Biol.* 5 (1985) 3610.
- [36] V. Sarraemegna, F. Talmont, A. Demange, P. Milon, *CMLS Cell. Mol. Life Sci.* 60 (2003) 1529.
- [37] B.C. McCusker, S.E. Bane, M.A. O'Malley, A.S. Robinson, *Biotechnol. Prog.* 23 (2007) 540.
- [38] B. Wade, A. Espagne, M.-A. Persuy, J. Vidic, R. Monnerie, F. Merola, E. Pajot-Augy, G. Sanz, *J. Biol. Chem.* 286 (17) (2011) 15252.
- [39] M.E. Van der Rest, A.H. Kamminga, A. Nakano, Y. Anraku, B. Poolman, W.N. Konings, *Microbiol. Rev.* 59 (2) (1995) 304.
- [40] J. Tschopp, R. Schekman, *J. Bacteriol.* 156 (1) (1983) 222.
- [41] R.P. Longley, A.H. Rose, B.A. Knights, *Biochem. J.* 108 (1968) 401.