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Phosphorylcholine Based Amphiphilic Polymers for the Solubilization of Integral Membrane Proteins

par

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Thèse présentée à la Faculté des études supérieures
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Abstract

Amphiphilic polymers, known as amphipols, have emerged as promising candidates to replace surfactants for the solubilization and stabilization in water of integral membrane proteins (IMPs). The limited portfolio of amphipols currently available has motivated this research project, which was aimed at developing new amphipols. Phosphorylcholine-based amphiphilic polymers were prepared to investigate their capacity as amphipols. Four random copolymers of n-octylacrylamide, *N*-isopropylacrylamide, and *N*-phosphorylcholine-*N*'-ethylenedioxybis(ethyl)acrylamide (PC) were synthesized and their ability to solubilize IMP was assessed using bacteriorhodopsin (BR), cytochrome *b6f*, and outer membrane protein A (OmpA) selected as model integral membrane proteins. The ability of the PC- based polymers to keep integral membrane proteins (IMP's) soluble was tested and compared with a polyacrylate-based amphipol in terms of trapping efficiency and stability. The amphipol/IMP ratios required to maintain IMPs soluble in water were determined and the size dispersity of the polymer/IMP complexes was measured. The stability of the complexes upon storage in neutral aqueous buffer of various salt concentration, in solutions of acidic pH (pH 5.0), in the absence of salts and in saline solutions was evaluated. The interactions in water between surfactants used for the extraction of IMPs from the membrane and the polymers (PC-based polymers and polyacrylate-based polymers) were studied as function of surfactants concentration by dynamic and static light-scattering (DLS and SLS), capillary electrophoresis (CE), and isothermal titration calorimetry (ITC). The formation of polymers/detergents mixed micelles and the composition of the mixed micelles were studied at different amphiphiles concentrations. The formation of such mixed micelles may affect the stability and the catalytic activity of IMPs. Furthermore, the exchanges of amphipols with detergent onto protein and vice versa were investigated. Thermodynamic and mechanistic study of each step of the exchange was performed by isothermal titration calorimetry (ITC) to determine the energy of conversion of protein/detergents complexes into protein/amphipols complexes and vice versa, and to assess the rate of amphipol-detergent exchange. The results of the whole study show that phosphorylcholine-based amphipols are promising tools for the solubilization and stabilization of integral membrane proteins.

Keywords: Solubilization; Amphipols; Integral membrane proteins; Phosphorylcholine; Amphiphilic macromolecules; Detergent; Mixed micelles; ITC.

Résumé

Les polymères amphiphiles, connus sous le nom d'amphipols, sont candidats prometteurs pour remplacer les surfactants pour la solubilisation et la stabilisation dans l'eau des protéines transmembranaires. Le portfolio limité des amphipols disponibles nous a conduits à développer un nouveau amphipol. Des polymères amphiphiles contenant le groupement phosphorylcholine ont été préparés pour étudier leur capacité en tant qu'amphipols. Quatre copolymères du n-octylacrylamide, *N*-isopropylacrylamide, et de *N*-phosphorylcholine-*N'*-ethylenedioxybis(ethyl)acrylamide (PC) ont été synthétisés et leur habilité à solubiliser des protéines transmembranaires a été évalué en utilisant bacteriorhodopsine (BR), cytochrome *b6f*, et la protéine membranaire A (OmpA) comme des protéines transmembranaires model. La capacité des ces polymères à maintenir en solution aqueuse non détergente ces protéines transmembranaires sous forme de complexes hydrosolubles a été examinée et comparée en terme d'efficacité et stabilité avec des complexes formées avec des amphipols à base des polyacrylates. Les rapports amphipols/IMP exigés pour maintenir ces protéines membranaires solubles dans l'eau ont été déterminés et la dispersité en taille des complexes polymères/IMP a été mesurée. La stabilité des complexes avec le temps dans un milieu aqueux neutre, dans des solutions de pH acide (pH 5.0), en l'absence des sels et dans des solutions salines a été évaluée. Les interactions dans l'eau entre des surfactants, normalement utilisés pour l'extraction des protéines transmembranaires, et les polymères (à base PC et à base polyacrylate) ont été étudiées en fonction des concentrations de surfactants avec plusieurs techniques, notamment la diffusion de lumière dynamique et statique (DLD et DLS), l'électrophorèse capillaire (EC), et le titrage calorimétrique isotherme (TCI). La formation des micelles mixtes polymères/détergents et la composition des ces micelles mixtes ont été étudiées en présence des différentes concentrations des amphiphiles. En fait, la formation de tels micelles mixtes peut affecter la stabilité et l'activité catalytique des protéines transmembranaires. Les échanges entre amphipols-détergents sur la surface des protéines transmembranaires et vice versa ont été effectués. Une étude thermodynamique de chaque étape a été réalisée par titrage calorimétrique isotherme (TCI) pour déterminer l'énergie de la conversion des complexes protéine/détergents en complexes protéine/amphipols et vice versa, et pour évaluer le taux d'échange amphipols-détergents. Les résultats de cette étude prouvent que les amphipols à base phosphorylcholine sont prometteurs pour la solubilisation et la stabilisation des protéines transmembranaires.

Mots-clés : Solubilisation; Amphipols; Protéines transmembranaires; Phosphorylcholine; Macromolécules amphiphiles; Détergent; Micelles mélangées; ITC.

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List of Abbreviations

Å	Ångström
A_2	Second virial coefficient
A8-35, A8-75, A34-35, A34-75	Polyacrylate based amphipols (“A”stands for Anionic, “8” and “34” refer to the molar mass and “35” and “75” are the percentage of free carboxylate groups)
AA	Acrylic acid
AIBN	Azo-bisisobutyronitrile
ANS	Anilino-naphthalene sulfonic acid
APol	Amphipol
ATP	Adenosine triphosphate
ATRP	Atom transfer radical polymerization
Boc-Ala-MBHA	Alanine methylbenzydrylamine
BR	Bacteriorhodopsin
^{0}C	Degree Celsius
C22-43, C60-44, C60-30, C45-68	Phosphorylcholine based amphipols (“C” stands for cationic, the digit adjacent to the letters refers to the molar mass (in kg.mol^{-1}), and the second digit is the molar percent of phosphorylcholine residues)
Ca^{2+}	Calcium ion

C8E4	n-Octyltetraethyleneoxide
C_8H_{17}	n-Octylacrylamide
$CaCl_2$	Calcium chloride
cal	Calorie
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CE	Capillary electrophoresis
CFTR	Cystic fibrosis transmembrane conductance regulator
cic	Critical interaction concentration
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CHAPSO	3-(cholamidopropyl)dimethylammonio-2-hydroxy-1-propane
$C_{10}M$	octyl pentaoxyethylene
cm	Centimeter
CMC	Critical micellar concentration
CRP	Controlled radical polymerization
CTA	Chain transfer agent
CTAB	Cetyltrimethylammonium bromide
cyt	Cytochrome
D	Diffusion coefficient
°	Degree

DAGK	Diacylglycerol kinase
DDM	n-Dodecyl-beta-D-maltoside
DHPC	1, 2-dihexanoyl-sn-glycero-3-phosphocholine
DLS	Dynamic light scattering
DM	n-decyl-beta-D-maltoside
DMF	Dimethylformamide
DMPC	Dimyristoylphosphatidylcholine
dn/dc	Refractive index increment
DNA	Deoxyribonucleic acid
E	Electric field strength
E. *coli*	*Escherichia coli*
μ_e	Electrophoretic mobility
EPR	Electron paramagnetic resonance
ε	Extinction coefficient
f	Frictional coefficient
F_b	Buoyant force
F_c	Centrifugal force
FACCE	Frontal analysis continuous capillary electrophoresis
FApol	Apol graft with a fluorescent dye (<1%)
FDA	United States Food and Drug administration

Fe_2S_2	Iron sulfur
FS	Fluorinated surfactants
g	Gram
GPC	Gel permeation chromatography
GPCRs	G-protein coupled receptors
H	Histamine
h	Hour
ΔH	Enthalpy
HFS	Hemifluorinated surfactants
HLB	Hydrophile-lipophile balance
HMCMP	Hydrophobically modified carboxymethyl pullulan
IBS	Irritable bowel syndrome
IMP	Integral membrane protein
ISP	Iron-sulfur protein
ITC	Isothermal titration calorimetry
J	Joul
k	Boltzmann's constant
K_A	Ligand concentration producing half occupation
K_B	Binding constants
K_d	Dissociation constant
K^*	Optical parameter

kDa	Kilodalton
kg	Kilogram
L	Liter
[L]	Ligand concentration
LDAO	N, N-dimethyldodecylamine-N-oxide
LPD	Lipopeptide detergent
LS	Light scattering
μ	Micro
m	Mass
M	Meter
M_n	Number average molecular weight
min	Minute
mM	Millimolar
Mw	Weight average molecular weight
n	Reaction stoichiometry
NaCl	Sodium chloride
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Monobasic sodium phosphate
Na_2HPO_4	Dibasic sodium phosphate
NaOH	Sodium hydroxide
NIPAM	N-isopropylacrylamide
nm	Nanometer

NMP	Nitroxide-mediated polymerization
NMR	Nuclear magnetic resonance spectroscopy
OG	Octyl glucoside
OmpA	Outer membrane protein A
OTG	n-Octyl-beta-D-thioglucoside
$P(\Theta)$	Particle scattering function
PC	Phosphorylcholine or N-phosphorylcholine –N'-[ethylenedioxybis(ethyl) acrylamide]
PDI	Peptitergent
PEO	Polyethylene oxide
pK_a	Ionization constant
PM	Purple membrane
psi	Pound force per square inch
q	Charge
r	Distance from the center of rotation
R	Ratio
$<r_g^2>$	Radius of gyration
R_h	Hydrodynamic radius
ρ	Density
$R(\Theta)$	Excess intensity of the scattered light at given angle (Θ)
RAFT	Reversible addition-fragmentation chain transfer polymerization

rpm	Revolutions per minute
s	Second
ΔS	Entropy
SDS	Sodium dodecyl sulfate
SLS	Static light scattering
Θ	Fraction of ligand binding sites occupied
TFA	Trifluoroacetic acid
THAM	Tris(hydroxymethyl)-acrylamidomethane copolymers
THF	Tetrahydrofuran
TMS	Trimethylsilane
t-OmpA	Transmembrane domain of outer membrane protein A
UV	Ultra violet
V	Volt
υ	Velocity
η	Viscosity
V_s	Partial specific volume of the particle
vis	Visible
ω	Angular velocity
λ	Wavelength
wt	Weight

A fact is a simple statement that everyone believes. It is innocent, unless found guilty. A hypothesis is a novel suggestion that no one wants to believe. It is guilty, until found effective.

Edward Teller

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THE AIM OF THE PRESENT STUDY

The ultimate goal of this study is to develop novel macromolecules effective in increasing the solubility of integral membrane proteins (IMPs) in aqueous media and to improve their stability in aqueous media. The study involves the design, characterization and development of polymeric surfactant capable of solubilizing IMPs to a high extent and eventually to maintain them in their native form in the absence of a lipid bilayer for extended periods of time, in the presence of salts and over a wide range of pH.

To achieve this objective, phosphorylcholine-based amphiphilic polymers were prepared and characterized. Bacteriorhodopsin (BR), cytochrome *b6f*, and OmpA were the IMP's chosen to test the synthesized phosphorylcholine-based polymers.

Chapter one presents an overview on integral membrane proteins, their types, their properties, the strategies used to increase their solubility in aqueous media, and the methods and techniques used throughout the work described in this thesis.

Chapter two contains a detailed study of the performance of four phosphorylcholine based amphiphilic polymers, having different chemical composition, in solubilizing IMPs and keeping them stable in aqueous solution compared to polyacrylate-based amphipols currently employed.

"*Complexation of integral membrane proteins by phosphorylcholine-based amphipols*" (2007) Biochimica et Biophysica Acta 1768, 2737-2747.

Chapter three reports a thermodynamic analysis of the interactions between the surfactants usually used for the solubilization of IMPs from the phospholipid bilayer and the polymers reported in this study using three techniques: light scattering (DLS and SLS), isothermal titration calorimetry (ITC), and capillary electrophoresis (CE). Fitting models were also employed to analyze the binding energy and the binding isotherms between free detergent and polymer-bound detergent.

"*Enthalpy of interaction and binding isotherms of non-ionic surfactants onto micellar amphiphilic polymers (Amphipols)*" (2007) Langmuir, 23, 6, 3025-3035.

Chapter four investigates the energy of formation and of exchange of amphiphile in complexes between various detergents, amphipols and proteins. The analysis of the exchanges and competitions involved in mixtures of amphipol, detergents and IMP was conducted using the regular mixing theory.

"*Enthalpies of exchange between detergents and amphipols at the surface of integral membrane proteins*". Journal of the American Chemical Society (JACS), to be submitted.

Chapter five presents a general discussion on the results obtained in the three studies.

Chapter six shows the perspectives of this study.

Appendix A and B, contains some supplementary data including the synthesis and the characterization of the synthesized phosphorylcholine-based amphiphilic polymers.

CHAPTER ONE

GENERAL INTRODUCTION

An overview on integral membrane proteins, their types, their properties, the strategies used to increase their solubility in aqueous media, and the methods and techniques used throughout the work described in this thesis.

Summary

Integral membrane proteins have important cellular roles and functions in living systems. They are involved in passive and active transport processes and take part in processes such as ion translocation, electron transfer, and transduction of extracellular signals. Others are receptors for viruses. IMPs comprise 20-30% of the proteins encoded in the genome of cells (von Heijne *et al.*, 1998). Therefore, they are highly relevant to human health. The malfunction of IMPs induces many diseases, such as hypertension, diabetes, cancer, cystic fibrosis, heart failure and so on. Thus, they are the most important target in drug development. They are currently targeted by over than 50% of the medicines in the market (Teller *et al.*, 2001).

Unfortunately, rather little information is available on their structure (Popot & Engelman, 2000; White & Wimley, 1999) because they tend to denature upon removal from the membrane. The difficulties arise from their hydrophobic nature. For spectroscopic and crystallographic studies, IMPs need to be transferred into aqueous solution. Therefore, a major challenge in pharmaceutical research is to obtain integral membrane proteins in a functional, solubilized, and monodisperse state in aqueous media.

It has been proposed to replace the lipid surrounding the hydrophobic domain of IMP with amphiphilic molecules. The hydrophobic domains of the amphiphiles will bind to the transmembrane surface of the IMP, while their hydrophilic domains provide a polar water-soluble exterior. The success of such systems depends on many physicochemical properties of the amphiphiles, including their structure, their

composition, their hydrophile/hydrophobe balance, their critical micelles concentration…..etc. The success also depends on the methodologies used and on the selection of appropriate conditions (pH, salt concentration, temperature,..). All these parameters will be discussed in details in this chapter.

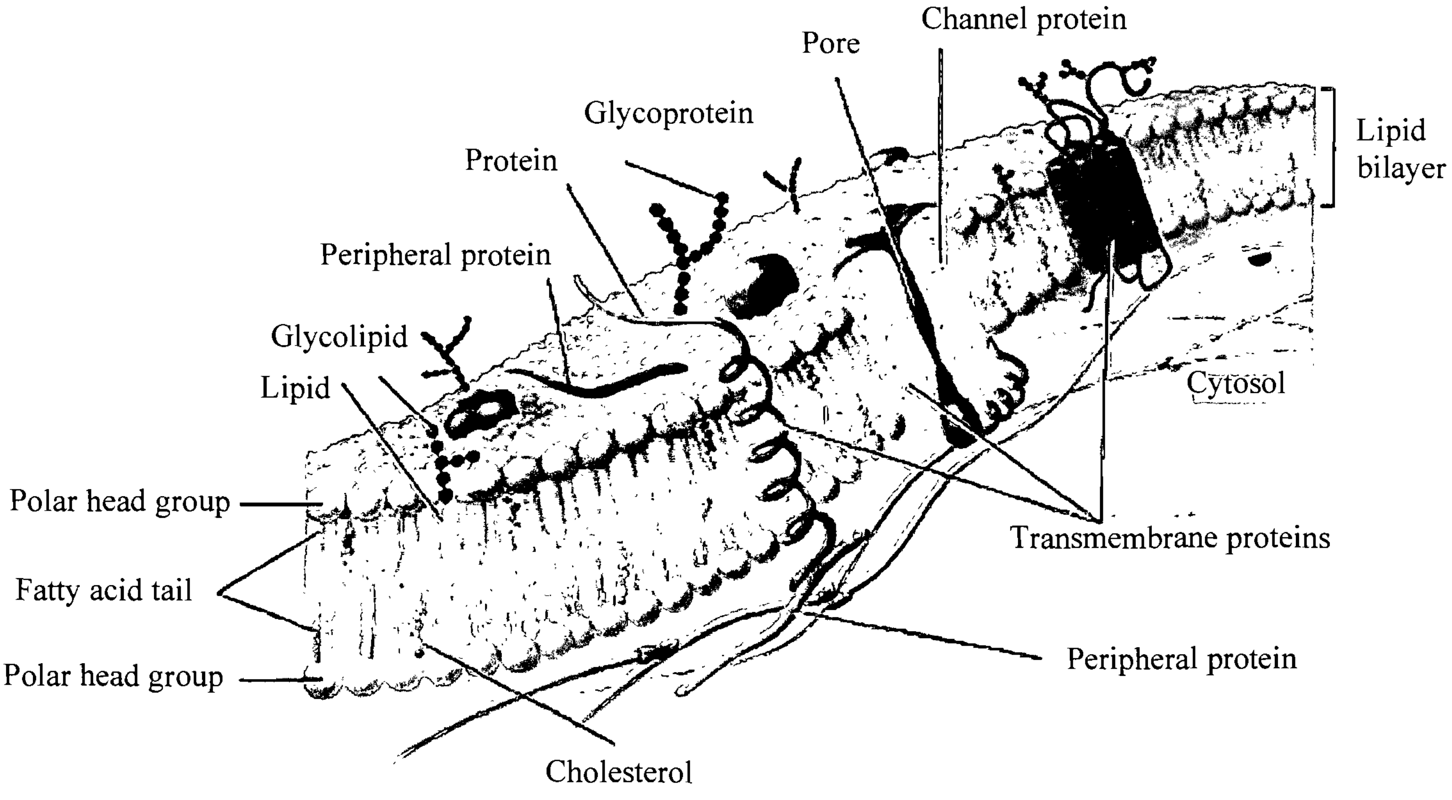
This chapter is divided into three parts. The first part focuses on the IMP used in this study, describes the different types of IMPs and concentrates on the strategies used for their solubilization and stabilization in aqueous media. The second part introduces the hypothesis of this project and the new polymer structure proposed for membrane protein research. Part three contains a description of the methods, techniques and fitting model used in this thesis.

This review will address many issues that might be useful to manipulate integral membrane proteins in aqueous solution. This will help to develop new drugs for many non-curable diseases.

1.1. Biological membrane

Cells and organelles are enclosed by the so-called biological membrane or lipid bilayer. Biological membranes are supramolecular assemblies that are composed of lipids and specific proteins (Figure 1.1). In addition, membranes contain carbohydrates linked to lipids and proteins.

Lipids are defined as any fat soluble molecules. They are esters of fatty acids, which are long hydrocarbon chains with terminal carboxylates. Like surfactants, lipids consist of a hydrophilic (water loving) "head" and a hydrophobic (water hating) "tail". When a fatty acid is placed in water, structures are formed that maximize the interactions of the hydrophilic heads with water and minimize the interactions of the hydrophobic tails with water. At low lipid concentrations, a monolayer is formed at the air-water interface, with hydrophilic heads associating with water molecules and hydrophobic tails pointing towards the air. As the concentration of lipid is increased, the surface area available for monolayer formation is reduced, leading to the formation of alternative structures depending on the lipid and the conditions. Compounds that have a relatively large head group and small tail group form spherical structures known as micelles. Compounds that have approximately equal-size heads and tails tend to form bilayers instead of micelles. In these structures, two monolayers of lipid molecules associate tail to tail, thus minimizing the contact of the hydrophobic portions with water and maximizing hydrophilic interactions.



www.ipam.ucla.edu

Figure 1.1: Schematic representation of a lipidic bilayer.

Different types of lipid head group exist, which differ in charge, size, shape and their capacity to form hydrogen bonds. The fatty acid chains usually contain an even number of carbon atoms, typically between 14 and 24, and they may be saturated or unsaturated. Indeed, there are different classes of lipid [e.g phospholipids, sphingolipids, lysophospholipids, isoprenoids, glycolipids, free fatty acids, ceramides, triglycerides, cholesterol and cholesterol esters], which in turn are divided into subclasses, each of which comprises a large number of different lipid types. For example, phospholipids include phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol. Furthermore, phosphatidylethanolamine can have different fatty acids and the number of different phosphatidylethanolamine types can be very high.

The amphipathic feature of lipids enables the formation of biological membrane that has continuous double layers of 4 to 5 nm thickness with a hydrophilic outside and a hydrophobic inside (Gorter *et al.,* 1997). The tight interactions between lipid tails result in the membrane acting as a boundary that is impermeable to most water-soluble molecules. Lipids of the membrane have diverse biological functions. In addition to being building block of the biological membranes, they participate in energy storage, thermal insulation, and surface protection in some organisms.... etc. The presence of lipids is also essential for the correct functioning of the membrane by providing specific packing properties and for processes such as membrane fusion and fission (de Kruijff *et al.*, 1997). Some lipids have highly specialized functions as regulators of metabolic activities (steroid hormones), blood pressure, or body temperature... etc.

Membrane proteins are proteins associated with the membrane of a cell or an organelle. They perform the major functions in membranes. Membrane proteins are classified into two groups, based on the strength of their association with the membrane: (i) transmembrane proteins (or integral membrane proteins (IMPs)) and (ii) peripheral membrane proteins. Integral membrane proteins span the entire lipid bilayer, and they interact with the hydrocarbon chain of lipids by hydrophobic interactions between their hydrophobic parts and the hydrophobic interior of the lipid bilayer. In contrast, peripheral membrane proteins are bound to the membrane surface or to integral proteins by a combination of hydrophobic, electrostatic, and other non-covalent interactions.

Cell membranes play a crucial role in homeostasis, which is the ability to maintain steady chemical balance in a changing environment, and in the biological communication between the inside and the outside of a cell, between different cells and between different organelles in one cell. Indeed, they possess active surfaces with a wide diversity of functions. As soon as they stop working, cell death ensues.

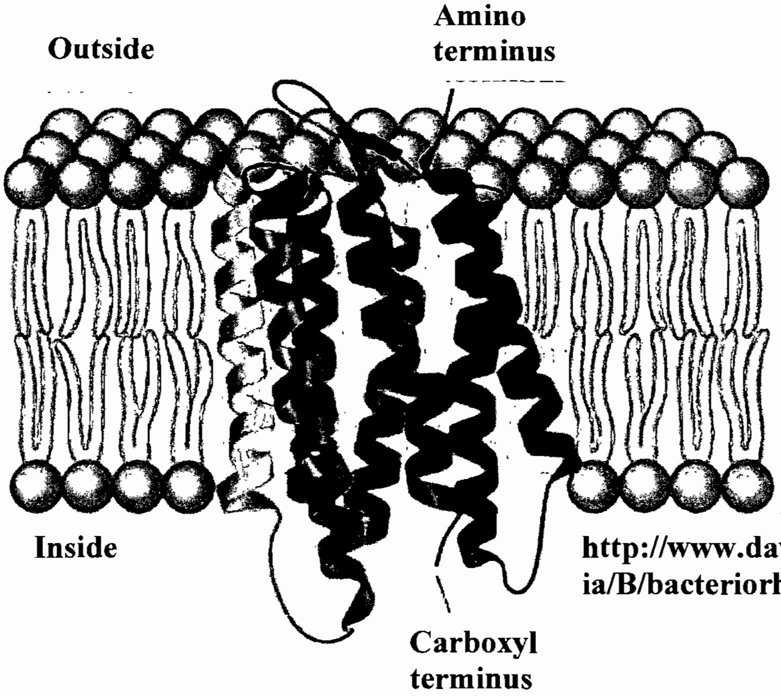
1.2. Transmembrane proteins

Transmembrane proteins are proteins that cross the biological membrane from the internal surface to the external surface (Figure 1.1). They fold in a manner such that their hydrophobic domain resides in the oily core of the membrane, while the hydrophilic domains protrude into the aqueous environment inside and outside the cell. There are two classes of transmembrane proteins depending on their folding motif: the alpha-helical and the beta-barrel (Popot & Engelman, 2000;

Subramaniam & Henderson, 2000; Schulz, 2002.). They will be described briefly in this section.

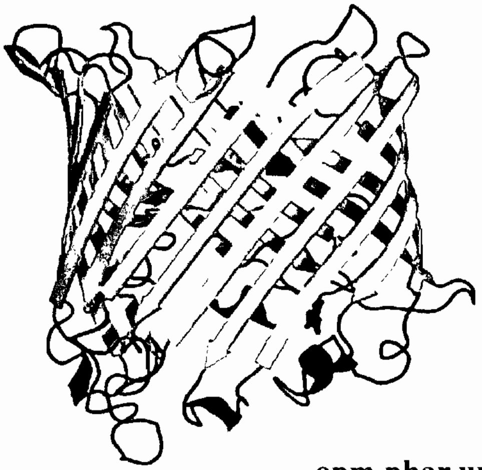
Integral membrane proteins of the alpha-helical class are present in all types of biological membranes including the outer membrane. They are of central importance in a wide range of vital cellular functions, such as photosynthesis, transport of ions and molecules across the membrane, attachment points for skeletal structures of the cell, and secretion of proteins. They form very hydrophobic transmembrane α-helices. The α helices run generally perpendicular to the membrane plane and connections are formed between neighboring helices ends (Figure 1.2) (Bowie *et al.*, 1999).

In another class of integral membrane proteins, the membrane spanning segments consist of β-strands organized in a barrel. A beta barrel is a protein fold that contains a series of beta sheets, typically arranged in an antiparallel fashion (Figure 1.3). The strands contain alternating polar and hydrophobic amino acids, so that the hydrophobic residues are oriented into the interior of the barrel to form a hydrophobic core and the polar residues are oriented toward the outside of the barrel exposed to the water. Other membrane proteins containing beta barrels have the reverse pattern, with hydrophobic residues oriented toward the exterior where they contact the surrounding lipids, and hydrophilic residues oriented toward the interior pore.



http://www.daviddarling.info/encyclopedia/B/bacteriorhod.html

Figure 1.2: Representation of the α-helical region of a transmembrane protein (Bacteriorhodopsin).



opm.phar.umich.edu/families.php?superfamily=71

Figure 1.3: Representation of the β-barrel transmembrane protein (Porin) from the bacterium *Salmonella typhimurium*, viewed from the side.

Beta barrels proteins exist as monomers, dimers, or trimers with 8-22 beta strands forming the barrel boundary. Their functions include active ion transport, passive nutrient uptake, membrane anchoring, membrane-bound enzymes and defense against the attack of pathogens. The barrel structures are commonly found in proteins that span cell membranes and in proteins that bind hydrophobic ligands in the barrel center. They are located in the outer membranes of bacteria, mitochondria and chloroplasts. Approximately 2–3% of the genes in gram-negative bacterial genomes encode beta barrels structure (Wimley *et al.*, 2003).

1.3. Transmembrane proteins used in this study

1.3.1. Bacteriorhodopsin

Bacteriorhodopsin (BR) is found in the photosynthetic system of the *Halobacterium salinarium*. In its native form, the BR molecule is located in a cell membrane commonly called the purple membrane (PM). It is the only protein constituent of the purple membrane and represents 2/3 of its total mass. The presence of BR is crucial to the life of the bacteria in an oxygen-deficient environment. The BR molecules function as light-driven proton pumps, which transport protons across the cell membrane (Lanyi *et al.*, 1995). This generates a proton gradient, which in turn produces an electrochemical potential used by the bacteria to synthesize adenosine triphosphate (ATP). This is the fundamental energy conversion process in biological cells. Bacteriorhodopsin has been characterized extensively in terms of its structure and functions and serves as model IMP. It is a starting structure for developing tools to handle transmembrane proteins in aqueous solution, for understanding the structure and function of membrane proteins and for modeling many medically important protein receptors.

BR has a molecular weight of 26 kDa. It is arranged in a two-dimensional crystal lattice naturally present as part of the plasmic membrane of the bacteria. It contains seven alpha helices (Figure 1.4 A) that enclose an all-*trans* retinal chromophore linked via a protonated Schiff base to the residue Lys216. This chromophore allows the BR molecule to absorb light, resulting in a photochemical reaction of the retinal from the all trans form to the 13-cis isomer and initiating a photocycle resulting in the transport of a proton across the membrane (Mathies *et al.*, 1991; Lanyi *et al.*, 1993; Lanyi *et al.*, 1998). The bound retinal chromophore, which has a broad absorption band centered at ~554 nm, gives a purple color to BR (Figure 1.4 B). Denaturation of BR is accompanied by the release of retinal, and consequently the color of BR becomes yellow and an absorption band appears at 382 nm indicating the presence of free retinal (Figure 1.4 B). Therefore, the stability of BR can be easily monitored by analyzing changes in its absorption spectrum.

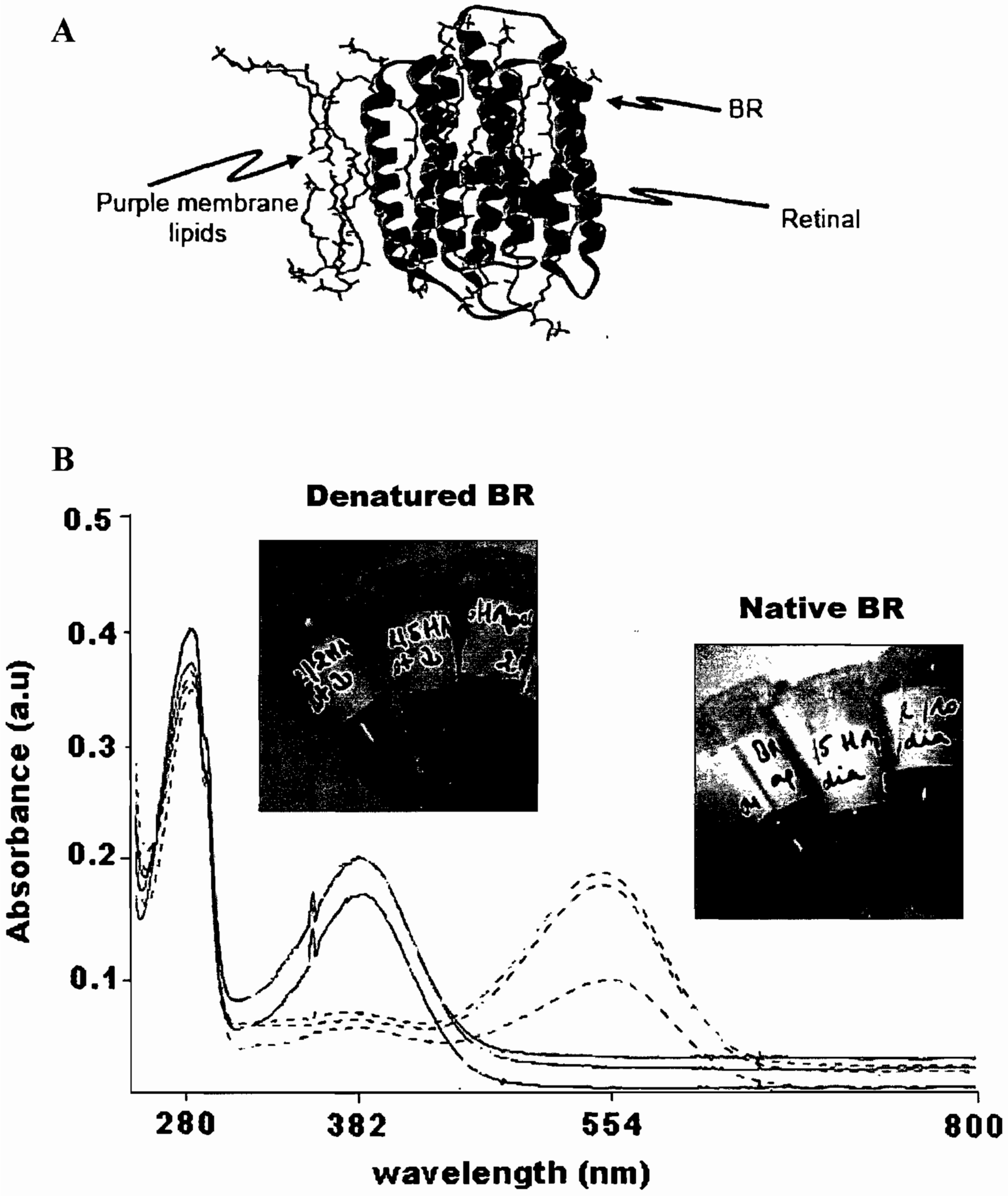
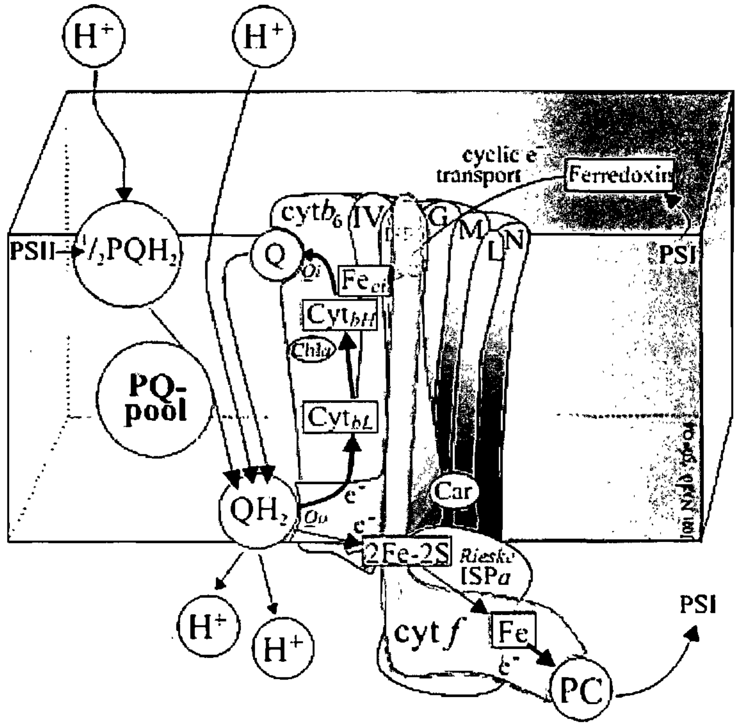


Figure 1.4: (A) Structure of bacteriorhodopsin, including the photosensitive retinal molecule. (B) UV-vis absorption spectra of native bacteriorhodopsin (BR) and denatured BR, as well as their colors. The figure is taken from http://www.e-mep.org/emeplab/Chalmers/23Popot.pdf.

1.3.2. Cytochrome b6f

The cytochrome b_6f complex, found in the mitochondrial respiratory system and in membranes of purple photosynthetic bacteria, such as *cyanobacterium M. laminosus* (Kurisu *et al.*, 2003) and green alga *C. reinhardtii* (Stroebel *et al.*, 2003), is involved in the electron transport during oxygenic photosynthesis. It occupies a central position in the electron-transport chain, accepting electrons from the photosystem II reaction center and donating them to the photosystem I reaction center that reduces ferroxidin and nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Figure 1.5). This process generates a transmembrane electrochemical proton gradient for adenosine triphosphate synthesis, providing the energy needed for other cell processes.

The cytochrome *b6f* complex (molecular weight of 217 kDa) is dimeric, and contains a core structure of three subunits: cytochrome b, the Rieske iron sulfur protein (ISP), and cytochrome f, which are arranged in an integral membrane-bound complex (Figure 1.5). Cytochromes f and b_6 are attached to the membrane by one transmembrane helix and four transmembrane helices, respectively. This dimeric structure has been revealed to be the functionally active form of the complex (Huang *et al.*, 1994). According to the crystal structure determinations (Kurisu *et al.*, 2003; Stroebel *et al.*, 2003), the monomeric unit (molecular weight of 108 kDa) of the cyt *b6f* complex contains eight subumits: four large subunits: cyt f, cyt b6, the Rieske iron-sulfur protein (ISP), subunit IV, and four small subunits (called also hydrophobic sticks): PetG, PetL, PetM, and PetN.



www.bio.ic.ac.uk/.../psIIimages/cytb6f.html

Figure 1.5: Representation of cytochrome *b6f* as well as the electron transport chain across the membrane.

Each monomer has 13 trans-membrane helices, 4 for the cytochrome b6 core of the complex, 3 for the subunit IV, and 1 for each small subunits. In addition, the *b6f* monomer contains seven groups: four hemes, one Fe_2S_2 cluster, one Chlorophyll a (Huang *et al.*, 1994) and one ß-carotene (Zhang *et al.*, 1999).

1.3.3. Outer membrane protein (OmpA)

The outer membrane protein A (OmpA) from *E. coli* is a monomeric membrane protein with 325 residues. Integral outer membrane proteins fold into antiparallel β-barrels (Figure 1.6). OmpA is one of the major Omps of *E. coli* with ~100,000 copies/cell (Koebnik *et al.,* 2000). It is also abundant in Gram-negative bacteria and it is an important target in defense against bacterial pathogens (Weiser & Gotschlich, 1991; Soulas *et al.*, 2000). Its main function is to maintain the structural integrity of the cell surface. It contains a transmembrane N-terminal domain and a water-soluble periplasmic C-terminal domain, which is believed to interact with the peptidoglycan layer and provide a physical link between the outer membrane and the cell wall (Koebnik *et al.*, 2000). It has been observed that a mutation in OmpA leads to spherical cells that cannot survive outside well-balanced osmotic conditions (Sonntag *et al.*, 1978). OmpA is also important for bacterial conjugation (Ried & Henning, 1987), and can serve as receptor for phages and colicines (Chai & Foulds, 1974; Sonntag *et al.*, 1978; Wang, 2002). It forms ion channels in planar lipid bilayers, facilitating the transport of solutes across the membrane (Sugawara & Nikaido, 1992; Sugawara & Nikaido, 1994; Stathopoulos, 1996; Arora *et al.*, 2000; Zakharian & Reusch, 2005).

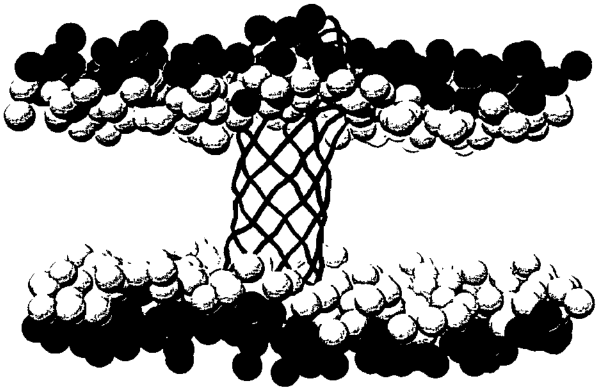


Figure 1.6: Representation of the transmembrane domain of the outer membrane protein A (OmpA) embedded in a lipid bilayer: the blue antiparallel sheet is the outer membrane protein beta-barrel, the blueish spheres are the carbons of the PC head groups, the red spheres are oxygens and the red spheres are phosphorus atoms.

1.4. Roles and functions of transmembrane proteins

Since transmembrane proteins participate activity to fundamental cell functions such as energy transduction, cell to cell communication, transfer of nutrients and drugs, adhesion, tissue formation, impairment of their functions may affect severely human health. Many genetic diseases, such as cystic fibrosis, Alzheimer's disease, some forms of diabetes result from mutations in membrane proteins. Therefore, IMPs are the target for most of the drugs currently in medicinal use.

A large number of extensively investigated transmembrane proteins are relevant to human health, so they become targets in pharmaceutical research. For example, G-protein coupled receptors (GPCRs), a large family of integral membrane protein, play important roles in a variety of fundamental biological processes. It has been found that GPCRs have seven sequence motifs with calculated high hydrophobicity (Attwood *et al.*, 1994). They act as cell surface receptors responsible for the transduction of a diversity of signals into a cellular response. Because of the importance of their roles and their link to a number of hereditary diseases (Stadel *et al.*, 1997), the search for therapeutic agents acting via GPCRs has attracted much attention as potential targets for drug development (Flower, 1999). In fact, it is estimated that over 50% of all modern drugs are targeted at GPCRs (Gudermann *et al.*, 1995). Notable examples of drugs acting via GPCRs, including **Zyprexa**, **Clarinex**, **Zantac**, and **Zelnorm** are presented in table 1.1.

Table 1.1. Marketed drugs targeted via GPCRs.

Name	Chemical name	Receptor
Atrovent	Ipratropium	Mixed muscarinic antagonist
Axid	Nizatidine	H_2 antagonist
Betaloc	Metoprolol L	$\beta1$ antagonist
Buspar	Buspirone	5-HT_{1a} agonist
Cardura	Doxazosin	α_1 antagonist
Claritin	Loratadine	Antihistamine H_1 antagonist
Cozaar	Losartan	AT_1 antagonist
Gaster	Famotidine	H_2 antagonist
Heitrin	Terazosin	α_1 antagonist
Imigran	Sumatriptan	5-HT_1 agonist
Lupron	Leuprolide	LH-RH agonist
Pepcidine	Famotidine	H_2 antagonist
Prepulsid	Cisapride	5-HT_4 ligand
Prostap	SR Leuprorelin	LH-RH agonist
Risperdal	Risperidone	Mixed D_2/5-HT_2 antagonist
Serevent	Salmeterol	β_2 agonist
Tagamet	Cimetidine	H_2 antagonist
Tenormin	Atenolol	β_2 antagonist
Ventolin	Salbutamol	β_2 agonist
Zantac	Ranitidine	H_2 antagonist
Zoladex	Goserelin	LH-RH agonist
Zyprexa	Olanzapine	Mixed D_2/D_1/5-HT_2 antagonist
Zyrtec	Cetirizine	Antihistamine H1 antagonist

Zyprexa is the drug name for Olanzapine. It is used for the treatment of psychiatric disorders, such as schizophrenia and bipolar disorder. The exact mechanism of action olanzapine is not known. It may work by blocking receptors for several neurotransmitters in the brain. It binds to alpha-1, dopamine, histamine H-1, muscarinic, and serotonin type 2 (5-HT2) receptors. It is Eli Lilly's top selling drug (32% of Eli Lilly's $14.6 billion revenue in 2006). It was approved for medicinal use by the FDA in 1996, as a new class of antipsychotic drugs without the side effects of the existing drugs. Other members of this class of drugs include clozapine (Clozaril), risperidone (Risperdal), aripiprazole (Abilify) and ziprasidone (Geodon). **Clarinex** is an antihistamine drug. Antihistamines are often used to help relieve the symptoms of seasonal and year-round allergies (itchy and watery eyes, sneezing and runny nose) caused by the exposure to dust, pets, and allergens coming from trees, grasses, or weeds. They act by blocking the action of histamine (H1), a substance that is produced by the immune system during an allergic reaction. **Zantac** (ranitidine) belongs to the class of H2-blockers, that blocks the action of histamine on stomach cells, thus reducing stomach acid production. It is used to treat and prevent the recurrence of ulcers and to treat heartburn, acid indigestion, or acid stomach. **Zelnorm** (tegaserod) is an oral medication for the treatment of constipation and constipation-predominant irritable bowel syndrome (IBS) in women. IBS is a chronic gastrointestinal disorder (constipation or diarrhea) characterized by periodic abdominal pain or discomfort and altered bowel function.

Another examples of integral membrane proteins relevant to human health are cystic fibrosis transmembrane conductance regulator (CFTR) and human erythrocyte anion exchanger 1 (band 3) in which mutations result in diseases. The removal of phenylalanine 508 in CFTR results in a processing defect and symptoms can arise as a result of the disruption of the flow of salts and water across the epithelial membranes (Hwang *et al.*, 1999). This alteration causes heavy, thick, and sticky secretions. As an example, when lung cells do not secrete enough fluid, a thick, sticky mucus accumulates, impairing breathing and promoting bacterial infection (McCarty, 2000). This malfunction of the epithelial membrane results in cystic fibrosis. Cystic fibrosis affects the entire body and limits growth, breathing, digestion, and reproduction, causing disability and early death. Currently, there is no cure for cystic fibrosis. However, many drug classes have been developed to treat this disease and to allow patients to live longer. The treatment depends on the severity, stage, and progression of the disease and complications of the disease such lung infection.

Band 3 is the major membrane protein of erythrocytes, which binds membrane skeletal proteins, glycolytic enzymes, and hemoglobin and transports various kinds of anions. Band 3 provides the anion channel of the erythrocyte plasma membrane. It facilitates the movement of the chloride and bicarbonate ions across the erythrocytes membrane. It is also involved in membrane stability and acid-base regulation of the blood (Wang *et al.*, 1994; Peters *et al.*, 1996). The mutation of band 3 results in the reduction of the integrity of the erythrocyte membrane, alters the shape of the cell, and induces hereditary spherocytosis.

These integral membrane proteins are three of a wide range of medically relevant proteins. This denotes that drugs targeting integral membrane proteins are profitable, as well as fruitful area of pharmaceuticals research. High resolution information on the structure of IMPs would help in the development of treatment for IMPs related diseases using rational methods for drug design.

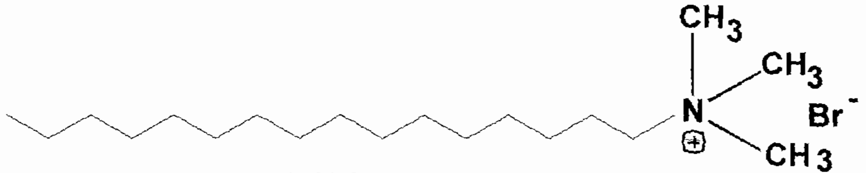
1.5. Solubilization of integral membrane proteins

Studies of IMP's in cells need to be complemented by measurements on IMP's extracted from their membrane and purified. When the membrane is removed during the solubilization and purification of the protein, the lipid bilayer must be replaced with an environment of similar characteristics to maintain the protein in its functional, folded state without any protein aggregation and precipitation. A major barrier for IMP solubilization is their high hydrophobicity, which makes them insoluble in aqueous media (Tanford, 1980), and the unavailability of a simple and reproductible procedure for isolating them in a monodisperse form that provides a native-like environment.

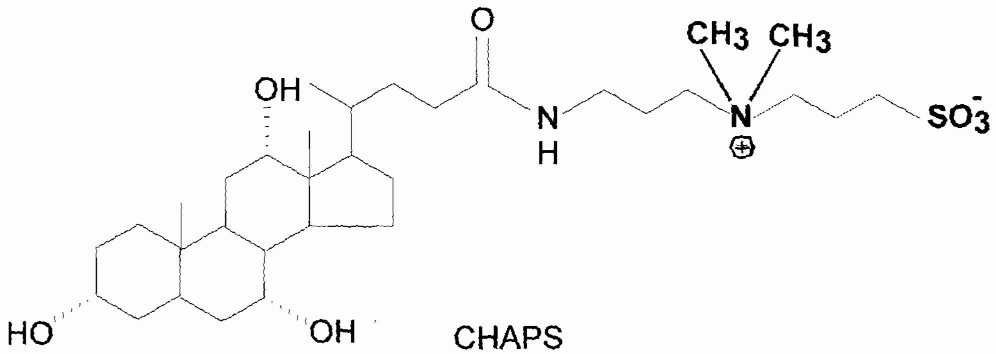
Detergents are commonly used to isolate, solubilize and manipulate integral membrane proteins for biochemical and physical characterization. Detergents are amphiphilic molecules (Helenius & Simons, 1975). They possess both hydrophobic and hydrophilic groups with a higher degree of hydrophilicity than most lipids in biological membranes. They have a hydrophobic moiety that binds to hydrophobic surfaces in a concentration-dependent manner (Rosen, 1978). Above a given concentration (the CMC), they associate into small, well-defined assemblies called micelles. Detergents can be broadly classified based on their charge. They can be anionic (e.g., sodium dodecyl sulfate (SDS) and bile salts such as cholate and deoxycholate), cationic (cetyltrimethylammonium bromide (CTAB)), zwitterionic (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)), and nonionic (polyoxyethylene family such as Triton X-100) (Figure 1.7). They bear respectively a negatively, positively, both negatively and positively, and non-charged head group that is attached usually to either alkyl group or steroid structure (Figure 1.7). Almost all ionic detergents, such as sodium dodecyl sulfate (SDS), trigger denaturation of IMPs after isolation from bilayer and cannot be used. Zwitterionic detergents tend not to induce IMP denaturation. For example, CHAPS is one of the used detergents in membrane chemistry (Chattopadhyay *et al.*, 2002). Nonionic detergents have been shown to solubilize transmembrane proteins without affecting their structure and are used to solubilize IMPs.

Some of the non-ionic detergents that have been shown to be useful in membrane protein functional and structural studies are alkyl glycosides, such as octyl thioglucoside (Figure 1.8) (Wiener, 2004; Raman *et al.*, 2006). For example, maltosides and glucosides bearing short chain alkyl (C8) have been successfully used to solubilize IMPs and help in their crystallization (Allen, 1994; Istvan *et al.*, 1995; Pebay-Peyroula *et al.*, 1995; Rosenow *et al.*, 2003). Also, longer chain glycoside, such as dodecyl maltoside (Figure 1.8), has been shown to stabilize G-protein coupled receptor (GPCR) (Jastrzebska *et al.*, 2006).

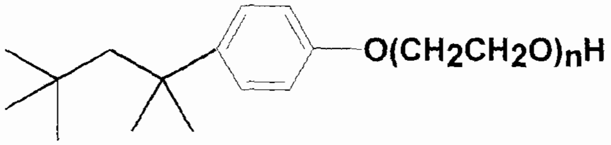
SDS



CTAB

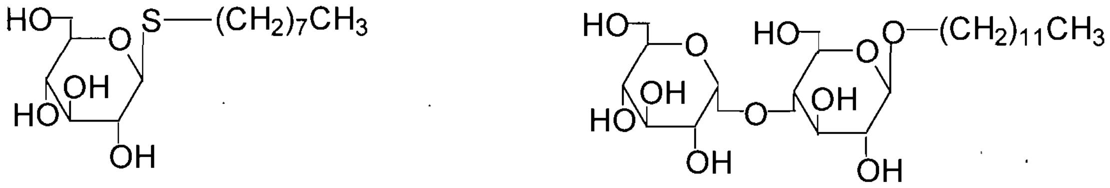


CHAPS



Triton X-100 (n=9-10)

Figure 1.7: Chemical structures of detergents of various charge types.



n-Octyl-beta-D-thioglucoside

n-Dodecyl-beta-D-maltoside

Figure 1.8: Structure of two commonly used detergents for functional and strucural studies of transmembrane proteins.

Short chain lipids detergents (C6-C8) as well as lysolipids show also detergent properties and are also useful for membrane protein biochemistry. They form water soluble aggregates in aqueous solutions. Several GPCRs were shown to remain functional after extraction with lysophospholipid micelles (Klammt *et al.*, 2005; Klammt *et al.* 2006). They were used in NMR studies of membrane proteins as in the case of cystic fibrosis conductance regulator (CFTR), which was solubilized in lysophosphatidylglycerol and investigated by NMR (Huang *et al.*, 1998; Krueger-Koplin *et al.*, 2004).

It is generally believed that during the extraction process that in a first step detergent monomers penetrate into the bilayer inducing formation of mixed lipid-detergent fragment (Figure 1.9A). Further detergent addition leads to bilayer dissolution and IMP solubilization (Figure 1.9B).

There is no single detergent for IMPs isolation. The choice of the detergent is one of the first decisions to be made in developing a protocol for isolating and purifying an IMP. There are several practical issues to consider while working with detergents and membrane protein. They will be discussed in details in this section.

a- Hydrophilic-Lipophilic balance (HLB)

The ability of a detergent to solubilize membrane proteins is believed to depend on the hydrophile-lipophile balance (HLB), which is a measure of the hydrophilic character of a detergent. It is defined as the weight percentage of hydrophilic *vs*. lipophilic groups present in a detergent (Helenius & Simons, 1975). It ranges between 0 and 40. In general, an HLB number smaller than 10 indicates

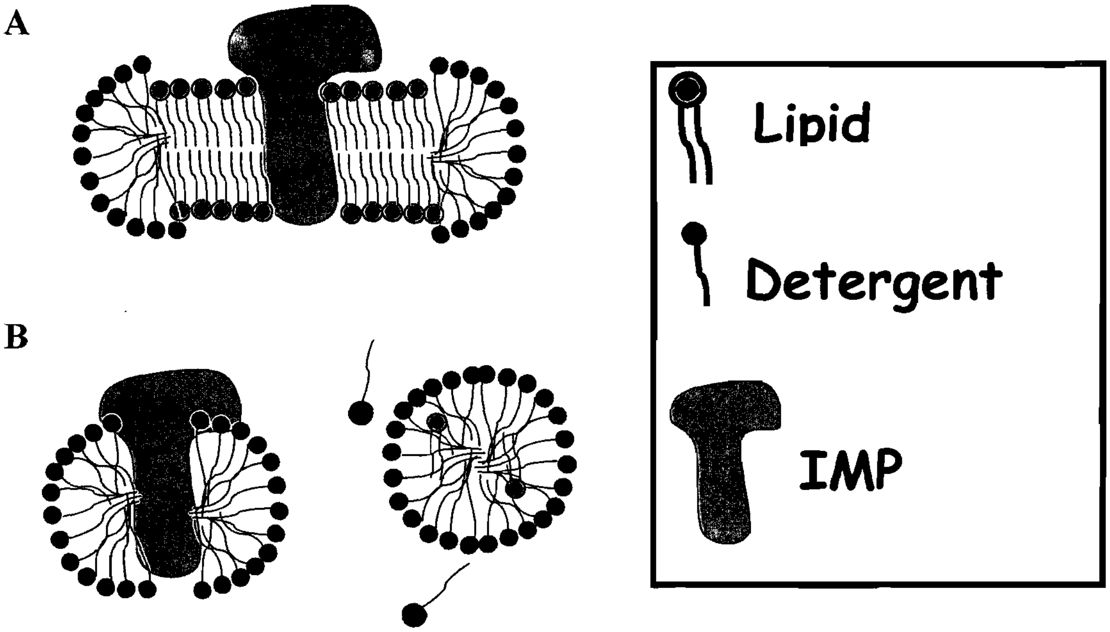


Figure 1.9: Representation of the solubilization process of IMP with detergent: (A) detergent monomers partition into the bilayer, (B) bilayer dissociation and IMP solubilization

that a detergent has low solubility in water, while an HLB number between 10 and 20 indicates that the detergent is soluble in water.

Detergents having a high HLB value (between 12-20) are recommended to extract membrane proteins in a non-denatured condition (Helenius & Simons, 1975). In studies with human adenosine A_3 receptor, a member of the GPCR superfamily, Berger *et al.* have shown that detergent with an HLB number between 12 and 15 correlates with extraction of A_3 and its high activity upon purification (Berger *et al.*, 2005). Three detergents that have shown to solubilize and maintain the stability of membrane proteins are presented in table 1.2. Many other studies have also shown that HLB values may be useful in selecting detergents for membrane protein extraction and purification (Dickie & Weiner, 1979; Nicholson & McMurray, 1986).

b- Critical micellar concentration (CMC)

The CMC is defined as the concentration of a detergent solution in water at which detergents molecules start to assemble into micelles (Figure 1.10). Isolated detergent molecules (monomers) exist in solution of lower concentration. As the detergent concentration increases beyond the critical micelle concentration (CMC), they self associate into small, well defined, non-covalently bound assemblies called micelles (Tanford, 1978). The micelles formation is described in terms of the hydrophobic effect (Tanford, 1980), an entropy-driven process. At surfactant concentration above the CMC, there exists an equilibrium between monomers and micelles and the amount of free monomer in solution remains constant as more detergent is added to the solution; only the concentration of micelles increases.

Table 1.2. Example of detergents used for membrane proteins solubilization.

Proteins	Detergent	HLB	Reference
Human adenosine A_3	Dodecyl maltoside	13.35	Berger *et al.,* 2005
B. *Subtilis* D-alanine carboxypeptidase	Triton X-100	13.50	Umbreit & Strominger, 1973
M. *luteus* phosphoacetylmuramyl pentapeptide translocase and succinate dehydrogenase	Triton X-100	13.50	Umbreit & Strominger, 1973

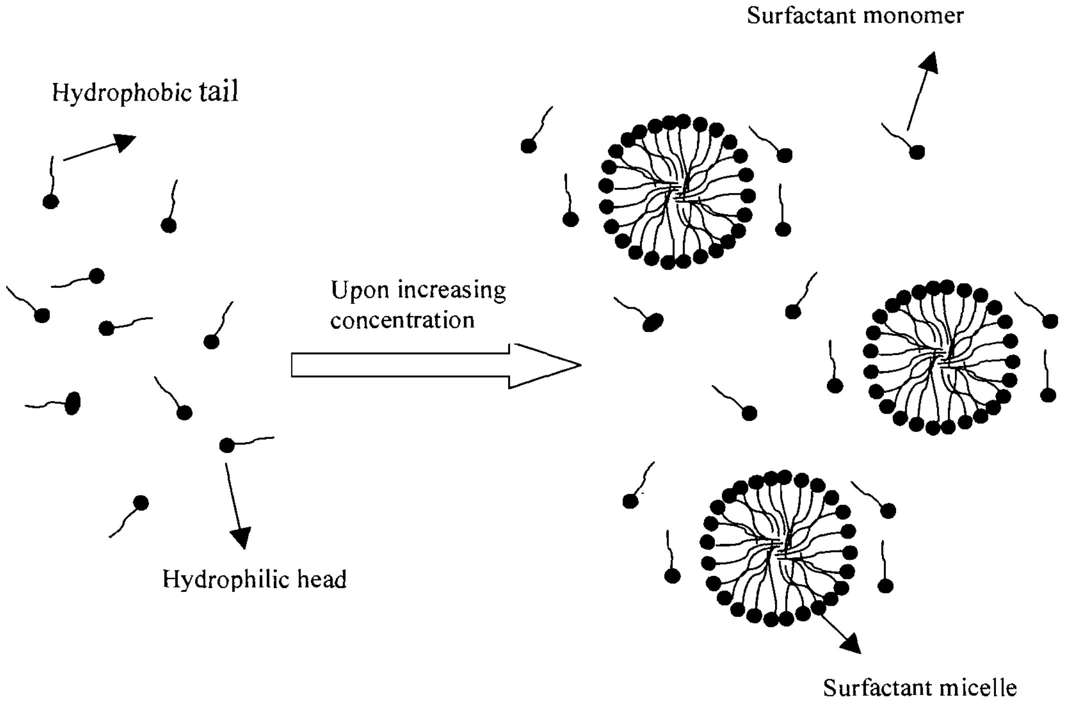


Figure 1.10: Micellization process.

Table 1.3. Properties of surfactants commonly used in membrane protein solubilization.[a]

Name	CMC (mM)	HLB	Aggregation number
Sodium n-dodecyl sulfate (SDS)	8	40	60
Sodium cholate	13	18	10
Sodium deoxycholate	5	16	10
3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)	1.4	15	10
n-Octyl-β-D-glucopyranoside (OBG)[b]	23	--	84
n-Octyl-β-D-thioglucopyranoside (OTG)[b]	9	--	100
n-Dodecyl-β-maltoside (DDM)[b]	0.15	--	98
n-Octyltetraethyleneoxide (C_8E_4)	8.2	--	82
Triton X-100	0.3	13.5	140

[a] Data from Jones, 1999 unless specified.

[b] Anatrace.

-- Unknown data.

There is a correlation between the CMC of a surfactant and the concentration required for solubilization (Rivnay & Metzger, 1982). The solubilization of most integral proteins is carried out with aqueous surfactant solution above their CMC. The detergent associates with the hydrophobic surfaces of membrane proteins to result in water-soluble protein-detergent complex (Figure 1.9) (Haneskog *et al*, 1996). Diluting IMP/detergent solution below the surfactant CMC triggers desorption of the surfactant and subsequently IMP aggregation and precipitation. The CMC of the detergent, the average micelle size and shape, as well as the size and shape of the detergent layer on a protein are function of the detergent. CMCs also vary as a function of the composition of the solvent (presence of salt, type of salt, concentration of salt, lipids, proteins, pH,..). For example, addition of salt is known to decrease the CMC of charged detergents such as SDS, since added salts tend to decrease the repulsion between charged head groups. This aids the formation of micelles at low concentrations of the detergent. The effect of salt on the CMC of uncharged detergents is less pronounced due to the absence of charge interactions. The length of the alkyl chain also affects the detergent CMC. Thus, short chain detergent has much higher CMC values than long chain detergents. The number of molecules in a micelle or aggregation number ranges from ten to several hundreds. It increases as the length of the hydrocarbon chain increases. Most detergents used for membrane proteins solubilization have aggregation number between 50 and 100 (le Maire *et al.*, 2000) (Table 1.3). Exceptions are some bile acid derivatives like CHAPS, CHAPSO, which have aggregation numbers of approximately 10 (Table 1.3).

c- Detergent removal

Detergents may interfere with certain analyses of IMPs and its excess must be removed after IMP solubilization. The detergent CMC plays a crucial role in determining which method should be used to remove unwanted detergent. Detergents with high CMCs, such as octylthioglucoside, are easily removed by dialysis and they are favored for membrane protein solubilization. In dialysis, detergent solutions are diluted below their CMC, so that micelles disintegrate into monomers, which can easily diffuse through the dialysis bag over time (Seddon *et al.*, 2004). Detergents with low CMCs are removed by adsorption on hydrophobic beads (Rigaud *et al.*, 1997). Detergent bound beads can be then removed by either filtration or centrifugation.

d- UV absorbance

A potential disadvantage of some surfactants such as the Tritons is the presence of an aromatic ring, which absorbs in the UV at 280 nm, the spectral range of side chains absorbance of proteins. This will hamper the UV detection of membrane proteins, which is a useful measure of their integrity. So, selection of a detergent with no UV absorption in the same range as protein (280 nm) is recommended.

e- Detergent purity

Other factors to consider comprise the method of synthesis, the method of purification and the chemical stability of the detergent. The synthesis may lead to side products (impurities), which can contaminate the detergent and induce membrane protein denaturation and aggregation. As an example, the presence of long chain alcohols, which are poorly soluble in aqueous solutions, may either form mixed micelles with the detergent or protein/detergent complexes.

f- Type of measurement

Structure and functional study of solubilized membrane proteins requires that the physical properties of the detergents and the resulting protein/detergent complex are compatible with the technique selected for this study. The techniques often used are nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallography.

The use of detergents for solution NMR study has been reviewed (Sanders & Sonnishsen, 2006). The ideal detergent should be a good mimic of the phospholipid bilayer in the sense that the structure adopted by the protein should be the same in the micellar system as when it is associated with bilayers. The tumbling of the detergent-protein complex in solution should be fast enough to yield spectra of sufficiently high resolution to permit spectral assignments and structurally interpretable data to be extracted. The detergent should result in a minimal increase in the size and molecular weight of the protein. The IMP/detergents complexes for structure determinations by NMR employ short chain detergents, such as sodium dodecyl sulfate (SDS), octyl glucoside (OG), octyl thioglucoside (OTG), 1, 2-

dihexanoyl-sn-glycero-3-phosphocholine (DHPC), and lysophospholipid, that form small assemblies in solution.

The detergents that are generally chemically well defined and nonionic such as, β-octyl glucoside, N, N-dimethyldodecylamine-N-oxide (LDAO), and pentaoxyethylene glycol monooctyl ether ($C_{10}M$) and all detergents that have moderate to high CMCs are best candidates for crystallization experiments. The detergents also should form monodisperse micelles with a small size (radius ~20-30 Å) and a fairly homogeneous spherical or ellipsoidal shape; thus the resulting protein/detergent aggregate may not have a size much larger than the protein alone. For example, cholates form large, heterogeneous micelle-like aggregates and were shown to be less successful in allowing membrane protein crystal growth (Helenius & Simon, 1975; Helenius *et al.,* 1979). The detergent should be strong enough to induce effective dissolution of all membrane lipids since the presence of lipids in the protein/detergent complex was shown to reduce the chances of crystallization (Garavito *et al.*, 1996).

f- <u>Type of protein</u>

The choice of a particular detergent depends on the membrane protein to be purified. For example, β-barrel proteins from bacterial outer membranes are usually flexible in term of extraction and solubilization protocols. They are compatible with a range of detergents, including moderately harsh small-micelle detergent that is favorable for NMR and X-ray crystallography. In contrast, most α-helical membrane proteins aggregate following extraction from membranes. Only few detergents such as dodecylmatoside (DDM) and octyl thioglucoside (OTG) have been used successfully.

g- Lipid removal

The protein can be extracted and purified in a way such that some lipids remain bound to it by using detergents that are not efficient lipid solubilizer or by minimizing the duration of detergent exposure. If needed, proteins can be completely stripped of lipids by using detergents that are highly efficient lipid solubilizers. This may be important in applications where homogenous protein preparations are required. In some cases, lipids supplemented to IMP/detergents to enhance protein activity and stability.

1.6. Peptide-like lipid

A- Peptitergent (PD1)

Peptitergents are 24-residue α-helical amphipathic peptides. They are synthesized using the same method of synthesis as for lipopeptides (described in the coming section) and are commercially available (SynPep, Dublin, CA, www.synpep.com). They have been designed to possess a hydrophobic surface of the same dimension as the lipid bilayer region of an integral membrane protein. The peptitergent helix contains a flat hydrophobic face that interacts with the hydrophobic domains of integral membrane proteins (Schafmeister *et al.*, 1993). They pack around the membrane proteins in a more rigid way and well-ordered manner. They have several advantages. Their chemical structure resembles common surfactant with similar CMC value and seems not to denature IMP. They are chemically and structurally simple with high solubility in water, and stability for a long period at room temperature.

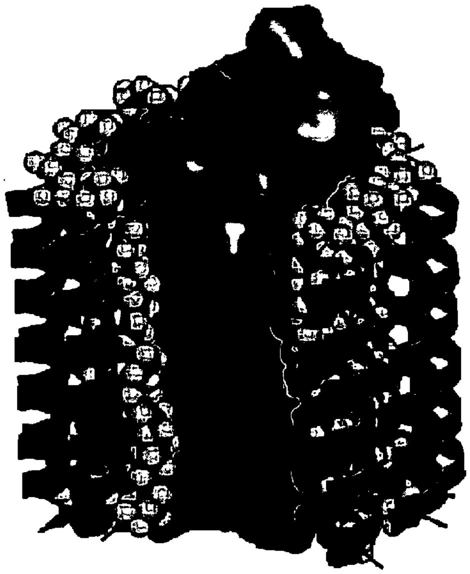
Peptitergents can maintain bacteriorhodopsin (85%) and rhodopsin (65%) soluble and native in aqueous solution for up to two days at 4 ^{0}C (Schafmeister *et al.*, 1993). However, they failed to maintain the solubility of the porin (Schafmeister *et al.*, 1993). The observations imply peptitergents are promising in term of solubilizing helical membrane proteins but not β-barrel structure membrane proteins.

B- Lipopeptide detergents (LPDs)

Lipopeptide detergents (LPDs) have been introduced by Privé and his colleagues (McGregor *et al.*, 2003) as novel solubilization agents to overcome limitations encountered by peptitergent. LPDs are 25 residue α-helical amphipathic peptides prepared using Boc- and Fmoc-based solid phase peptide synthesis methods via the sequential addition of Fmoc α-amino-protected monomers to a boc protected alanine methylbenzydrylamine (Boc-Ala-MBHA) resin (McGregor *et al.*, 2003). The terminal amino group is acetylated with acetic anhydride. The boc-protected δ-amino group of the two ornithine residues is then deprotected with 50% trifluoroacetic acid (TFA). The free carboxylic group is activated with o-(7-azabenzotriazol-1-yl)-1, 1, 3, 3-tetramethyluraniumhexafluorophosphate and coupled to fatty acids (ranging from 12-20 carbons in length). The products are then fully deprotected and released from the resin with trifluoromethanesulfonic acid.

The presence of alkyl acyl chains induces the self-assembly of LPD into micelles with small radius (~ 2.3 to 3.1 nm) and well-defined aggregation number (depends on the length of the alkyl chain). Unlike peptitergent, the critical micelle concentration (CMC) is extremely low (~10^{-6} M) and does not depend on the length of the alkyl chain of the fatty acid linked to the peptide. Lipopeptides were designed such as to share the properties of both the detergent and lipid bilayer. Consequently, they can form micelles like detergent, and they can accommodate uniform acyl packing like phospholipids. This design and the presence of a peptide allow them to assemble into cylindrical particles with an outer of α-helices exposed to the water and a hydrophobic core filled with the alkyl acyl chains. Furthermore, since they fold into a α-helix, the alkyl chains align with the hydrophobic domain of the IMP, so they generate small, more rigid, well-ordered complex with membrane protein (Figure 1.11). This would increase the stability of the complexes obtained and facilitate their crystallization.

LPDs are effective for the solubilization of α-helical membrane protein (bacteriohodopsin and the lactase permease) (McGregor *et al.*, 2003), as well as the *Escherichia coli* PagP protein, a β-barrel protein (Hwang *et al.*, 2002). They were shown to stabilize bacteriorhodopsin for over one month at room temperature at a pH of 7, but they were not stable at conditions, which are generally unfavorable for the storage of BR in solution, such as acidic pH (< 5.5) and the presence of salts. The stability of the LPD/proteins complexes depends on the alkyl chain length of the fatty acid and the concentration of the LPD.



http://xtal.uhnres.utoronto.ca/prive/projects.ht

Figure 1.11: Representation of a protein-lipopeptide detergent complex. The membrane protein is shown as a surface representation (green). LPD molecules are shown in ribbon representation for the peptide chains (red), which contact solvent, and space-filling for the alkyl chains (white), which contact the protein.

A small amount of aggregation was observed with storage in LPD having 20 carbons in length (higher limit) and at concentrations of LPDs less than 0.5 mM and more than 2.5 mM (optimal LPD concentration is between 0.5 and 2.5 mM).

LPDs are able to mimic the lipid bilayer environment and they are well suited for IMP crystallization and NMR spectroscopy study. However, their synthesis and purification is costly and difficult, which has limited their applications.

1.7. Fluorinated and Hemifluorinated surfactants (HFS)

The most frequently encountered problems of hydrogenated detergents are related to the dynamic nature of their micellization and interactions with IMPs (Breyton *et al.*, 1997). A solution for this problem could be a surfactant that has less affinity towards lipophilic surfaces and do not compete too much with lipids. Such surfactants will not be very efficient in releasing lipids and proteins directly from the membrane. The low detergency of such surfactant will allow a small amount of lipids to remain attached to IMP providing an environment that may keep the protein in its native structure.

Fluorinated surfactant (FS) and hemifluorinated surfactants (HFS) have been shown to have this property. They are highly tensioactive, hydrophobic but also lipophobic, they do not interact well with the alkyl chains of lipid due to weak van der Waals interactions, but also due to the bulkiness and the rigidity of the fluorinated region (Scott, 1958). As a consequence, they are lipophobic and cannot not solubilize biological membranes. Moreover, fluorinated alkyl chains interact less favorably with the transmembrane proteins surface than their hydrogenated counterparts. This makes them less efficient in removing the proteins directly from the lipid bilayer.

HF and HFS are usually obtained by linking by radical reaction either a fluorocarbon or an ethyl end-capped fluorocarbon chain onto the double bond of a hydrogenated detergent (Polidori *et al*, 2006).

The ability of FS and HFS to solubilize and stabilize integral membrane proteins have been tested on three model proteins of different type of structures (BR, OmpA and Cyt *b6f*) (Der Mardirossian & Lederer, 1996; Der Mardirossian *et al.,* 1998; Breyton *et al.*, 2004; Polidori *et al*, 2006). IMPs were extracted first using a hydrogenated detergent to keep them stable in aqueous solution, then the hydrogenated detergent was substituted with fluorinated detergent. BR was shown to be more stable in HF and HFS than in OTG, the best hydrogenated detergent used to solubilize BR. Also, *b6f* was kept soluble, active and stable in fluorinated and hemifluorinated surfactants. Its stability was much lower in hydrogenated surfactants compared to HFS or FS. *b6f*/hydrogenated detergent complexes inactivated rapidly upon storage. This suggests that the complexes with the hemifluorinated detergent have retained some lipids initially bound to them.

While the results obtained with HFS are promising, the use of this type of surfactants for protein purification and stabilization requires further study and investigation. The chemical and physico-chemical properties of these molecules, as well as their stability in aqueous media should be studied. Furthermore, their synthesis is difficult and costly. Protocols will have to be improved to produce them at satisfactory cost. Additionally, more details on the stability of the proteins are needed in order to improve their usefulness in membrane proteins study.

1.8. Bicelles

Bicelles (bilayered micelles) (Figure 1.12) are aqueous lipid-detergent assemblies in which bilayers are edge-stabilized by certain detergents (Sanders *et al.*, 1994; Sanders & Landis, 1995). Examples of bicelles include mixtures of dimyristoylphosphatidylcholine (DMPC) with dihexanoylphosphatidylcholine or with the bile salt derivative, 3-(cholamidopropyl)dimethylammonio-2-hydroxy-1-propane (CHAPSO) (Sanders & Prestegard, 1990; Sanders & Schwonek, 1992; Sanders, 1993). The properties of bicelles depend primarily on the q-value, which is defined as the molar ratio of lipid to detergent. At relatively high lipid concentration (10-20% by weight), bicelles with q = 3-4 have been used to examine the structure and the orientation of membrane proteins (Sanders & Prestegard, 1990; Sanders *et al.*, 1994; Sanders & Landis, 1995; Howard & Opella, 1996; Losonczi & Prestegard, 1998; Struppe *et al.*, 1998). Smaller bicelles with q = 0.5, which form an isotropic solution, have proved suitable for high-resolution structural studies of membrane-bound peptides (Vold *et al.*, 1997).

Bicelles have several advantages compared to micelles. For example, Sanders and Landis have shown that diacylglycerol kinase retains its activity in bicelles, but not in micelles (Sanders & Landis, 1995).

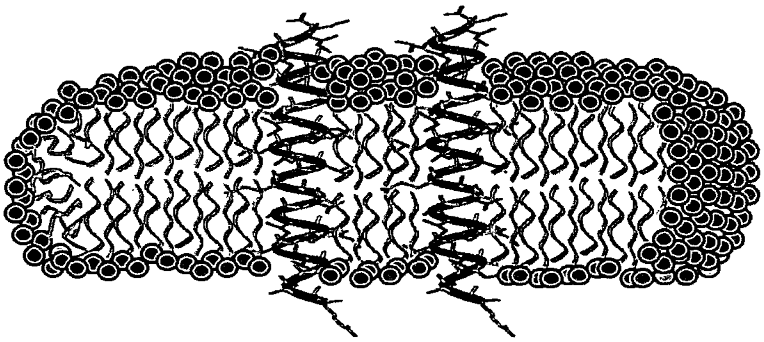


Figure 1.12: Schematic representation of a bicelle (in blue and dark yellow) containing an α-helical transmembrame protein (in red).

Diacylglycerol kinase (DAGK), an enzyme extracted from *Escherichia coli*, is an integral membrane protein that has three transmembrane spans. DAGK can be reconstituted in bicelles, where it remains catalytically active and stable for 48 h at 40 ^{0}C. Furthermore, BR was able to refold in both DMPC/CHAPS and DMPC/DHPC bicelles (Booth *et al.*, 1996; Booth *et al.*, 1997).

Another important development in the use of bicelles have been the demonstration that bacteriorhodopsin can be crystallized from bicelles (Faham & Bowie, 2002). Bicelles have also proven to be useful for NMR spectroscopy studies as a medium to gain access to structurally useful dipolar coupling and chemical shift anisotropy data of membrane proteins (Sanders & Landis, 1995; Bax, 2003).

In spite of their usefulness for some applications, the existing bicelles are not applicable to all studies such as those that must be carried out at room temperature or below (upon cooling bicelles undergo phase transition from liquid crystalline to gel), and the use of dilute bicelles mixtures for some studies; this could be sensitive to variations upon changing some conditions (pH, salt, batch of lipid used..). In fact, bicelles are more complex than the simple model of monodisperse bilayered systems (Sanders *et al.*, 2004) and additional characterization of the existing bicellar systems is needed. So this model remains a subject of investigation.

1.9. Amphipols

This approach proposed by Tribet *et al.* (Tribet *et al.*, 1996) consists of using a polymeric surfactant or amphipol, which provides multipoint attachment between the nonpolar side chains of the polymer and the transmembrane protein hydrophobic surface (Tribet *et al.*, 1996; Popot *et al.*, 2003). In general, amphipols are linear amphiphilic polymers with randomly distributed hydrophilic and hydrophobic units in specific amounts. They are believed to solubilize integral membrane proteins by wrapping around the protein hydrophobic transmembrane region and providing a polar water-soluble exterior (Tribet *et al.*, 1997). They form an interface between the solution and the protein's transmembrane region, to which they bind non-covalently but quasi-irreversibly. They are not effective in solubilizing membrane proteins from the membrane. However, when added to IMP/surfactant complex, they displace the surfactant to yield stable IMP/polymer complex (Figure 1.13). Thus, membrane proteins remain soluble in the absence of detergent, and become, considerably more stable (Popot *et al*, 2003). A typical protocol for the purification of transmembrane proteins starts with the solubilization of the membrane containing it with a detergent, followed by several purification steps carried out in the presence of the micelles of the same detergent. An amphipol is then added in excess. The concentration of the detergent is then lowered below its CMC. The detergent monomers are eliminated subsequently by adsorption onto Bio-Beads, by dialysis, or sedimentation of the complexes onto a sucrose gradient.

The ability of such amphipols to handle IMPs and keep them stable depends on many factors, including the type of amphipols, the composition of amphipols and the amount of amphipols added to IMP. Amphipols are low molecular weight highly flexible polymers, which can adapt to the small radius of curvature of protein surfaces and their irregularities (Popot *et al.*, 2003). The size of the polymeric micelles is small and similar to that of transmembrane proteins and the hydrophobic

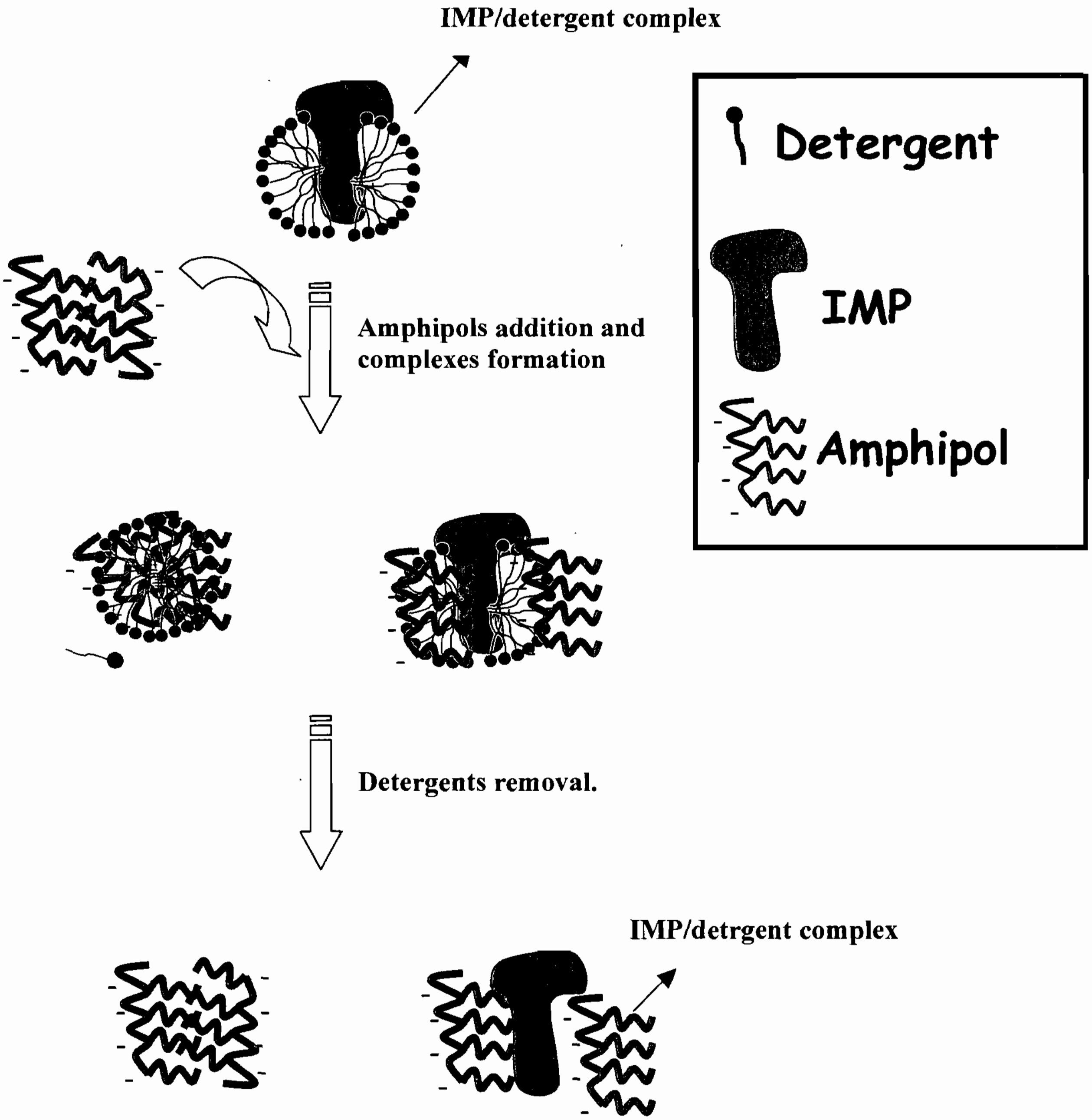


Figure 1.13: Representation of the transfer of the IMP from detergent to amphipols.

group is short to keep the polymer soluble in aqueous media. Many amphipol structures have been developed. They will be described in details in this section.

A- <u>Polyacrylate-based amphipols</u>

Polyacrylate-based amphipols are random anionic copolymers of acrylic acid, *N*-octylacrylamide and *N*-isopropylacrylamide (Figure 1.14). They are prepared by reacting commercially available poly(acrylic acid) with octylamine and isopropylamine to form amide bonds (Wang *et al.*, 1988). The hydrophobicity of the polymer is controlled by the amount of octyl groups, while the charge density can be adjusted by modifying the carboxylate grafting percentage. The starting polyacrylates has a polydispersity of ~2 (the polydispersity is the ratio of the weight average molecular weight over the number average molecular weight of the polymer). Several structures, having different monomers composition and polymer molecular weight, were synthesized by C. Tribet (Tribet *et al.*, 1996). The most commonly used polyacrylate amphipols is the one that has an average chain length of 70 monomers and an average molecular weight of 8 kDa. Fewer experiments were conducted with longer chains polymer (~300 monomers (Tribet *et al.*, 1996)) and shorter ones (~ 25 monomers (Gohon, 1996)).

The use of the amphipol designated as A8-35 ("A" stands for anionic, the number 8 refers for the average molar mass in kDa, and the number 35 is the percentage of free carboxylate groups), as IMP solubilization tool, has been described by Y. Gohon *et al.* (Gohon *et al.*, 2006).

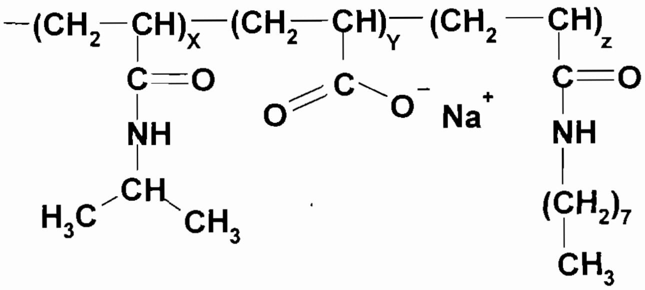


Figure 1.14: Structure of polyacrylate-based amphipols.

The size, the molecular mass and the size dispersity of amphipols assemblies were studied as a function of concentration and pH. They have shown, using different experimental techniques that A8-35 self-assembles into small, compact particles of well defined size. Each particle contains, on average, four A8-35 macromolecules. They noted that A8-35 forms two types of assemblies: small particles of 3 nm in radius and a minor population of large aggregates (~45 nm). The large aggregates can be removed by ultracentrifugation or gel filtration yielding 3 nm particles, which showed no tendency towards dissociation upon dilution down to 0.5 g/l or aggregation upon increasing the concentration up to 40 g/l. The size dispersity of the particles is narrow independently on the polydispersity in mass of the precursor polymer (having either a broad polydispersity of 3.1 or a narrow polydispersity of 1.3) used to prepare the amphipols. The size and size dispersity of the particles depends on the pH of the media. In alkaline media, particles of smaller size and narrow size dispersity were obtained. Upon lowering the pH below 6.8, the particles which become neutral and water insoluble in acidic media, start to aggregate and the size increase as well as the size dispersity. This is due to their weak acid nature (pK_a~ 6).

Polyacrylate based amphipols were used to stabilize four integral membrane proteins (Tribet *et al.*, 1996): (i) bacteriorhodopsin, (ii) a bacterial photosynthetic reaction center (RC), (iii) cytochrome *b6f*, and (iv) matrix porin. The performance of several polyacrylate based amphipols were tested: A8-35, A8-75, A34-35, and A34-75 ("A"stands for Anionic, "8" and "34" refer to the molar mass and "35" and "75" are the percentage of free carboxylate groups). After dilution below the CMC and centrifugation to remove the aggregated proteins, a complete precipitation was observed either in the absence of polymers or in the presence of the unmodified polyacrylates. In contrast, in the presence of modified polymers, amounts of native proteins were detected. The amount of the proteins trapped depends on the amount of polymers added. In fact, partial precipitation resulted from too low amphipol/protein ratio. The optimal amphipol/proteins ratio was found to vary from one protein to another, but for a given protein it was nearly the same for all the polymers studied.

In term of stability, BR, which is not very stable and denatures rapidly in detergent solution, remained stable for a period of time, which depends on the polymer compositions and the protein type. For example, with A8-35 no aggregation of the BR occurred for twelve days, while in the case of A8-75 about 25% of BR aggregated during the first two days. The activity of cytochrome *b6f* complexed by any of the polymers decreased rapidly with time and it appears that the complexes with detergent were more stable compared to that obtained with the amphipols. The two other proteins RC and porin matrix were highly stable in the presence of both polymers.

Polyacrylates amphipols, which carry weak acid groups, form soluble complexes with IMPs as long as the acid groups remain ionized. However, a decrease of solution pH below ~6.5 triggers aggregation of the amphipol (Gohon *et al.*, 2006) and IMP/amphipol complexes (Gohon *et al.*, 2007; Zoonens *et al.*, 2007). Also, an increase in ionic strength above a value of ~300 mM or the presence of calcium ions affect the colloidal stability of IMP/amphipols in neutral conditions

(Picard *et al.*, 2006), which limits the range conditions accessible for IMP purification, functional assays, and structure determination by NMR spectroscopy or crystallographic techniques. These observations have prompted research into alternative amphipol structures.

B-Polymers derived from Tris(hydroxymethyl)-acrylamidomethane (THAM)

Neutral amphipols such as tris(hydroxymethyl)-acrylamidomethane (THAM) copolymers (Figure 1.15) have been shown ability to stabilize IMPs in the same manner as the polyacrylates (Prata *et al.*, 2001). Polymers are prepared by free-radical copolymerization of THAM and derivatized-THAM monomers in the presence of a chain transfer agent (dodecanethiol) and an initiator (azo-bisisobutyronitrile (AIBN)) in boiling THF for 12 hours. The molecular weight of polymers and their hydrophobic/hydrophilic balance were varied using THAM-based monomers of various hydrophobicity (Pucci *et al.*, 1991; Polidori *et al.*, 1994). Prata *et al.* prepared a set of polymers 10-100 units long (Prata *et al.*, 2000), comprising either 15-18 or 25-28 mol % of hydrophobic groups.

Their ability to solubilize IMPs was tested using bacteriorhodopsin and cytochrome b_6f (Prata *et al.*, 2000). After solubilization with telomers, b_6f preserved its native state upon storage at 40 ^{0}C for over two weeks (as shown from its enzymatic activity). BR complexed with any of several telomers was also kept in native state.

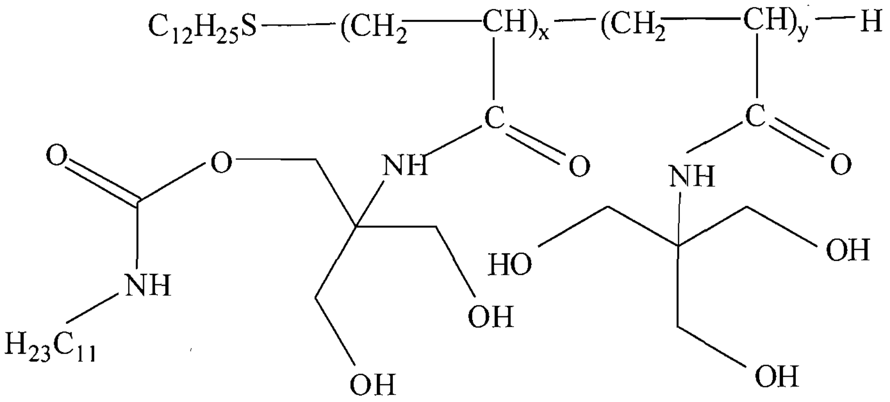


Figure 1.15: A nonionic amphipol obtained by random cotelomerization of hydrophilic and hydrophobic monomers derived from THAM (Prata *et al.*, 2001).

There is a correlation between polymer structure (polymer molecular weight, hydrophobic content, number of carbon of the alkyl group) and their efficiency to solubilize either BR or *b6f*. Polymers with a hydrophobic content of 24-30 mol% alkyl chains were more efficient than polymers containing 17-20% hydrophobes in maintaining low polydispersity of IMP/polymer complexes and a high enzymatic activity of the protein. At comparable degrees of hydrophobic modification, C_{11} alkyl chains were more efficient than C_7 at keeping BR and b_6f native. Long polymers (about 100 monomers) with low grafting degree (17%) had the same efficiency as shorter polymers (11-23 monomers) with a higher grafting degree (22-25%), at keeping *b6f* complex monodisperse, but much less at keeping its functionality. This indicates that an appropriate hydrophobicity is critical parameter rather than polymer molecular weight to control the efficiency of telomers.

C- PMAL-B

Another family of amphipols, PMAL-B, has been reported by J. K. Nagy. (Nagy *et al.*, 2001) (Figure 1.16). They are commercially available from Anatrace (Maumee, OH, USA). They have a C_{12} hydrophobic group and they are highly ordered in the sense that all repeating units of the polymer have four backbone carbon positions. The first two have carbonyl containing side chains (either two carboxyls or a carboxyl and an octylamide), followed by a carbon with no side chain, and the last carbon with a dodecyl side chain (Nagy *et al.*, 2001).

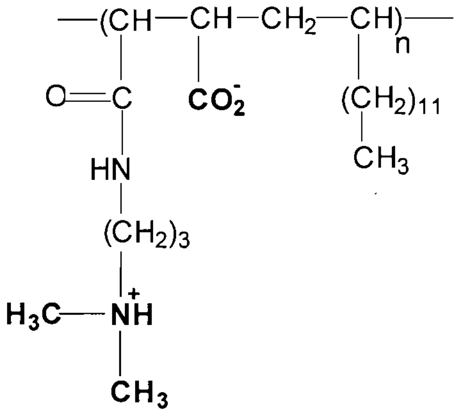
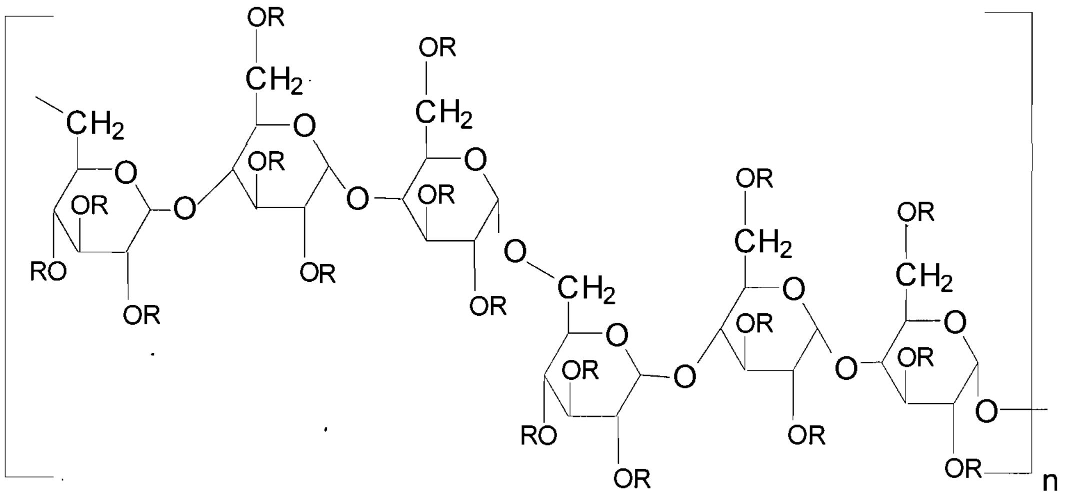


Figure 1.16: PMAL-B-100, an amphipol that is zwitterionic at neutral and basic pH and cationic at acidic pH (Nagy *et al.*, 2001).

Their degree of amidation was set to be 25% and their average molecular weight was 10 kDa based on the molecular weight of the precursor polymer (polydispersity was ~2) and the measured degree of derivatization. There exist three type of PMAL-B: PMAL-B-0, PMAL-B-50, PMAL-B-100. At neutral and basic pH, PMAL-B-100 is zwitterionic, containing both carboxylate and ammonium amide groups linked to contiguous units. They are cationic under acidic conditions. In the case of PMAL-B-50, only half of the repeating units are zwitterionic, while the other half consists of two contiguous carboxylate units. In PMAL-B-0, all repeating units have two carboxylates. The nature of the PMAL-B (100 % zwitterionic, 50% zwitterionic, or anionic) is a critical factor controlling the ability of a polymer to solubilize and stabilize IMPs. Only PMAL-B-100 yielded DAGK/polymer complexes exhibiting full activity in the absence of additional lipids or detergents. The concentration of PMAL-B-100 influences greatly the amount of complexed protein. For example, when the amount (weight) of PMAL-B-100 present is equal to that of complexed to the enzyme only 65% of proteins were obtained. The stability of DAGK in PMAL-B-100 was slightly low than that in DM micelles. DAGK activity was much lower when it was complexed with PMAL-B-50 (50% zwitterionic, 50% anionic). This was ascribed to the anionic nature of PMAL-B-50, as supported by the fact that DAGK's activity in PMAL-B-0 (fully anionic) was very low compared to PMAL-B-100 and PMAL-B-50.

D- Hydrophobically modified carboxymethylpullulans (HMCMPs)

HMCMPs, also called amphibiopols, are hydrophobically modified carboxymethyl-pullulans (Duval-Terrie′ *et al.*, 2003) (Figure 1.17), of medium molar mass (Mn~30 kDa), and narrow size distribution. A large set of amphibiopols, differing in the nature of their hydrophobic chain (octyl, decyl, dodecyl, and phenylpropyl group), in the hydrophobic modification ratio (number of hydrophobic groups per 100 anhydroglucose units), and in the charge ratio (number of ungrafted carboxymethyl groups per 100 anhydroglucose units), were synthesized by chemically modifying pullulan, which is a polysaccharide polymer consisting of maltotriose units connected by α-1, 6 glycosidic bond. The carboxymethyl groups were introduced to generate carboxymethyl pullulan (CMP). Then, carboxylic groups were coupled to hydrophobic chains (Duval *et al.*, 2001). Pullulans bearing different hydrophobic groups were tested and compared in term of their capacity to handle proteins from the outer membrane of the bacterium *Pseudomonas fluorescens* (OmpF) and bacteriorhodopsin (BR) in aqueous solution (Duval-Terrie′*et al.*, 2003). The polymers were able to extract the proteins directly from the membrane unlike the case for other amphipols. The solubilization of the proteins was influenced by the nature and the length of the hydrophobic chain. Decyl groups appeared to be the most suitable, providing a compromise between solubilization efficiency and polymer solubility. The polymer bearing phenylpropyl groups was not efficient solubilizer of the hydrophobes, probably due to their bulkiness, and the fact that they are shorter and less flexible than linear alkyl chains.



R= -H (pullulan)
$-CH_2-COO^- Li^+$
$-CH_2-COO-(CH_2)_{n-1}-CH_3$
or $-CH_2-COO-(CH_2)_3-C_6H_5$

Figure 1.17: Structure of hydrophobically modified carboxymethylpullulans (HMCMPs).

There exists an optimum degree of hydrophobic modification, for example 18% for polymers bearing decyl groups. The concentration of proteins solubilized increases initially with polymer concentration and then decreased at high polymer concentration. The most appropriate conditions for the solubilization of OmpF (68%) were found to be a concentration of 2g/l of polymer having 18% C_{10}. The optimal concentration of polymer may be different for other proteins. Ungrafted and C_8 grafted CMP were inefficient for protein solubilization.

In spite of the ease of chemical modification and composition modulation, the trapping efficiency of these amphipols is still low and further study and investigation are required.

1.10. Hypothesis of this work

Amphipols nanoparticles are widely used for membrane protein research. For this purpose, amphipols are required to have a good biocompatibility, a small micellar size, and the ability to maintain the native structure of the protein in the absence of a lipid bilayer over an extended period of time, in the presence of salts and over a broad pH range. None of the existing amphipol meets all of these criteria. The goal of this study is to design a polymer that has the same solutions properties of as current amphipols in terms of micelle size, solubility, and hydrophobicity, and promotes protein stability without the lipid bilayers over extended period of time, in the presence of salts and in acidic media.

An attractive approach to enhance the solubility of amphiphilic polymers in buffers of physiological salinity in a broader pH range involves the replacement of the carboxylate units of amphipols by units that are insensitive to pH and salts, yet are highly hydrophilic, such as several zwitterionic units, in which a cationic and an anionic groups are linked covalently and are known to possess this property (Nagy *et al.*, 2001; Kudaibergenov *et al.,* 2006). One of them is phophorylcholine, which is known to be biocompatible. Thus, it has been selected for this study. Using the design rules established for amphipols of the A8-35 class and the synthetic procedure of PC-containing amphiphilic polymers devised by Miyazawa and Winnik (Miyazawa &Winnik, 2002), we prepared PC-based amphipols and used them for transmembrane proteins solubilization and purification. They are random copolymers of n-octylacrylamide, *N*-isopropylacrylamide, and *N*-phosphorylcholine-*N'*-ethylene dioxybis(ethyl)acrylamide.

It is well known that phospholipids are essential constituents of biological membranes. Given this, there have been intensive efforts to prepare synthetic polymeric phospholipid analogues (phosphobetaines such as phosphorycholine derivatives). Phospholipid polymers are often insoluble in pure water due to the formation of intra- and interchain ion pair contacts resulting in ionically cross-linked networks. However, the polymers become very soluble in water upon the addition of monovalent salts; the ions penetrate in the polymeric ionic network, screen the net attraction between the polymer chains and promote solubility. Chain expansion also occurs upon addition of salt, the so-called antipolyelectrolyte effect. Current amphipols, being polyelectrolytes, repulse each other electrostatically in solutions of low ionic strength, but in solutions of higher ionic strength, the repulsion disappears, to be replaced by inter-chain attraction; this induces precipitation of the amphipols or protein/complexes with subsequent protein denaturation.

1.10.1. PC-based polymers

The phosphorylcholine head group is predominant among the phospholipids forming the bilayers that make up cell membranes. PC is zwitterionic (Figure 1.18). It contains an equal number of positively and negatively charged groups in a molecule thus maintaining neutrality within a wide pH range. PC-based polymers are of interest in many fields such as biochemistry, chemistry, polymer science and biomaterial science on the premise of achieving a good biocompatibility. They belong to the class of polybetaines, which differ from polyampholytes in that both cationic and anionic species are on the same monomer residue.

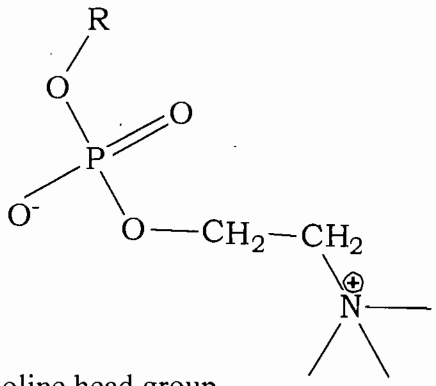


Figure 1.18: Phosphorylcholine head group

PC based polymers have been shown to increase the biocompatibility of materials onto which they were applied. They have been used in a series of medical devices, including stents (Zheng *et al.*, 1999), contact lenses (Kunzler & McGee, 1995), cardiopulmonary by pass equipment, biosensors (Chen *et al.*, 1993), thoracic drain catheters (Hunter & Angelini, 1993), and dialysis membranes (Campbell *et al.*, 1993). The PC modified polymers confer enhanced biocompatibility, reduced thrombogenicity, inflammatory response and bacterial adhesion and are capable of local drug delivery (Jeong *et al.*, 1997), including antibiotics, anti-inflammatory, hormonally active agents, nucleic acid derivatives and therapeutic proteins. One example of their application is as contact lenses component for which reduction of protein adsorption is extremely important as it helps to maintain comfort and visual clarity of the lenses and reduces the potential for infection (Franklin *et al.*, 1993; Kunzler & McGee, 1995). The zwitterionic nature of the PC headgroup ensures a high level of water through the lens. The water associates tightly with the PC in the lens, which results in less dehydration than in conventional hydrogel lenses.

Several groups focused their interest on the polymers with phosphorylcholine functional group (Nakaya *et al.*, 1989; Regen *et al.*, 1982; Chapman *et al.*, 1986). The hypothesis for using PC is based on the concept of biomimicry, in which phosphorylcholine copolymers create an interface that mimics the biomembrane, thus reducing undesired biological interactions. Surfaces with PC-polymers exhibit decreased protein adsorption in the same manner as cell membranes, which have a high PC content on their surface. PC-polymers can be synthesized in a number of ways. The first method for their synthesis developed by Nakaya and Li (Nakaya & Li, 1999). It involved the free radical polymerization of a methacrylate-based PC monomer obtained by reacting 2-hydroxyethylmethacrylate with 2-chloro-2-oxo-1, 3, 2-dioxaphospholane, followed by ring-opening of the intermediate alkoxyphospholane with trimethylamine. With this PC-based monomer (called

MPC), a wide range of homopolymers and copolymers with comonomers such methylmethacrylate or styrene (Kojima *et al.*, 1991) was synthesized, characterized and tested for biomedical and pharmaceutical application. The monomer is commercially available but not in North America. It is difficult to prepare and it has limited stability during purification. The MPC copolymers have shown excellent blood compatibility, suppression of protein adsorption and platelet adhesion (Ishihara *et al.*, 1992; Ishihara, 1997). Recently, Winnik and Miyazawa reported an alternate method leading to PC-polymers. The route involves a procedure where PC groups are linked to a preformed polymer via reductive amination of phosphorylcholine glyceraldehyde by primary amine groups attached to the polymer (Miyazawa & Winnik, 2002). The yield of the synthesis was very high and the purification of the resulting polymer was straightforward. They demonstrated a flexibility in polymer structure and composition. All the PC-modified polymers prepared by this route are cationic under the conditions of our measurements as a consequence of the presence of a secondary amine in the fragment linking the phosphorylcholine group to the polymer main chain.

The properties of PC-polymers in aqueous solution are controlled by the interactions of the zwitterions in aqueous and aqueous salt solution. PC units of the polymer have tendency to form ion pairs via the interaction of the cationic and anionic groups of neighboring monomer residues (intragroup) or head-to-tail stacking (intrachain) within single macromolecule and interchain ion contacts between neighboring macromolecules. This results in a cross-linked network and in low solubility of PC-polymers in pure water. However, they are highly soluble upon addition of salts. The electrolyte penetrates the ionic network, screens the net attractive electrostatic interactions between the polymer chains, resulting in chain expansion (as the inter and intra-chain association break up) and enhancing the solubility of the polymer. Another important feature of PC-polymers is that phosphorylcholine unit is pH insensitive and remains zwitterionic at any pH, so it will promote solubility of the polymer over an extended pH range.

Hydrophobically modified PC-polymers combine the behavior of zwitterions and amphiphilic polymers. They have been shown to form polymer assemblies in aqueous media. They were able to solubilize water-insoluble fluorescence probes and various hydrophobic drugs via adsorption by non-covalent bonding through hydrophobic interaction (Ishihara *et al.*, 1999). Generally, the solubilization capacity increases as the hydrophobic content of the polymer increases.

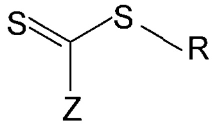
None of the PC-base copolymers available can be employed as amphipols, which, to be effective need to have controlled architecture and lipophilic/hydrophilic balance, as demonstrated previously on polyacrylates-based amphipols (Tribet *et al.*, 1996). Therefore, the synthesis of new PC-based copolymers is needed.

1.11. Method of synthesis

1.11.1. Reversible addition-fragmentation chain transfer polymerization (RAFT)

The living/controlled radical polymerization techniques (CRP) were developed to enable the synthesis of a wide range of polymers with well-defined architectures, compositions and functionalities by techniques readily applicable in common laboratory settings and industrial environment (Iovu & Matyjaszewski, 2003). Unlike conventional free radical polymerization, chain transfer agents are used to control the number of active radicals present in the system at any given time. As a result, the reaction generates polymers with predetermined molecular weights and narrow polydispersity. Three methods exist to perform controlled/living polymerization, atom transfer radical polymerization (ATRP) (Matyjaszewski & Xia, 2001), reversible addition-fragmentation chain transfer polymerization (RAFT) (Chiefari *et al.*, 1998), and nitroxide-mediated polymerization (NMP) (Gygi & Galli, 2005).

Among CRP techniques, the RAFT (reversible addition-fragmentation chain transfer) process was introduced in 1998 by the group of Rizzardo (Chiefari *et al.*, 1998). It consists of adding, in the polymerization medium, a chain transfer agent (CTA) (Figure 1.19). A large number of such compounds have been prepared by varying the structure of the Z and R groups (Figure 1.19). Z and R are classified in the literature based on three criteria: (i) their structure, (ii) the monomers that have been polymerized with the corresponding CTAs and (iii) the type of polymerization performed (solution, dispersed media, surface initiated, and copolymerization).



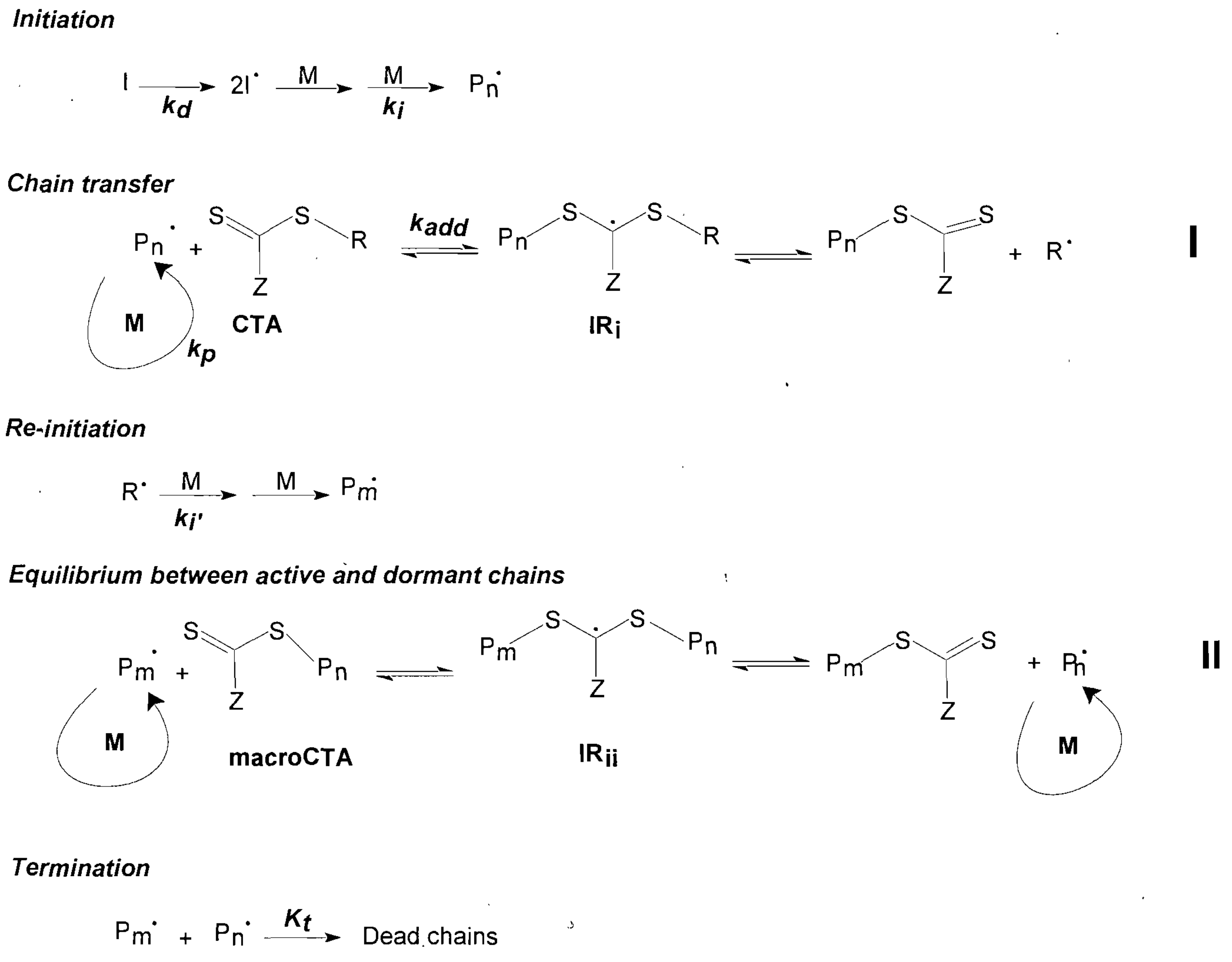
Z= aryl, alkyl, NR'$_2$, OR', SR'.

R= homolytic leaving group.

Figure 1.19: General structure of a thiocarbonylthio compound.

The general mechanism of RAFT polymerization is described in scheme 1. The free-radical is generated as in conventional free radical polymerization either by a thermoinitiator or a photoinitiator. For example, azo compounds such as 2, 2-Azo-bisisobutyronitrile (AIBN) have been used to generate radicals by heating to 65^0C or by UV illumination. The reaction starts between free radicals and monomers that lead to a growing species. The transfer between the active species and the thiocarbonylthio-containing CTA induces the formation of dormant species (equilibrium I in scheme 1) and the release of a radical, which is able to initiate a new polymer chain (re-initiation). The dormant species, which also bears a thiocarbonylthio function (macroCTA), induce reversible addition-fragmentation equilibrium (equilibrium II, in scheme 1). The active species can also propagate and terminate. However, the presence of a constant (low) amount of radicals at any given time, and thus the concentration of active species, provides a constant polymer growth and minimizes the termination reaction. In other word, the polymer chains successively pass from a dormant state to an active state during which monomers can be added, and grow in parallel during the polymerization.

Due to the homogenous chain growth, a good control of the molecular weight distribution can be obtained by RAFT. The average polymerization degree increases with conversion, while the polydispersity index remains low and theoretically decreases with conversion. The number-average molecular weight can be predicted from the conversion and CTA concentration.



Scheme 1. General mechanism of RAFT polymerization

It is given by the equation below:

$$M_n = \frac{[\text{monomer}]_0 \text{ x } M_m \text{ x } \%\text{conversion}}{[\text{CTA}]_0} + M_{CTA} \quad \textbf{(1)}$$

M_n: Number-average molecular weight

M_m: Monomer molar mass

$[monomer]_0$: Initial monomer concentration

$[CTA]_0$: Initial CTA concentration

M_{CTA}: CTA molar mass

Using RAFT polymerization, homopolymers, random and block copolymers and branched polymers with narrow polydispersities have been easily prepared. Smulders and Monteiro have copolymerized styrene and n-butylacrylates to produce well-defined core shell nanostrucures with controlled particle sizes and molecular weight distributions (Smulders & Monteiro, 2004). Scales *et al.* have used RAFT to graft a series of nonionic, cationic, anionic and zwitterionic homo- and block copolymers to gold particle. Xin and coworkers have reported the synthesis of zwitterionic block copolymers via RAFT (Xin *et al.*, 2005). Nevertheless, the field of application is still wide open, especially the choice of monomers and chain ends bearing specific side groups to introduce various functional groups to the polymer chains (Kujawa *et al.*, 2006). The RAFT technique will provide a versatile pathway to synthesize amphipols of controlled molecular weight and architecture.

1.12. Techniques used in this study

1.12.1. Velocity sedimentation (Ultracentrifugation)

Velocity sedimentation methods have been used over the past decades for the characterization of the particles size distributions and as a tool for detecting and quantifying aggregates in protein (Bridgman, 1942; Baldwin & Williams, 1950; Vinograd & Bruner, 1966; Scholte, 1968; van Holde & Weischet, 1978; Lechner & Mächtle, 1992; Stafford, 1992; Mächtle, 1999).

The size distribution of proteins and macromolecules to be used in biology and pharmacy needs to be determined as accurately as possible. Various techniques based on different principles have been developed for this task, including high resolution dynamic light scattering, and ultracentrifugation. Ultracentrifugation is the oldest of these techniques. It has been replaced by other techniques, which are faster and more precise. However, ultracentrifugation is still used in many applications for the following reasons. First, it is attractive for its theoretical simplicity and it can be applied directly for the separation of populations of different sizes, without the need for an interaction with matrices and surfaces. Second, this technique is experimentally powerful and versatile; the macromolecules can be studied in solutions and over a wide range of concentrations. Third, it yields useful characteristics such as size, molecular weight, size dispersity, sedimentation velocity, and shape with high precision (Hanlon *et al.*, 1962; Schachman *et al.*, 1962; Laue, 1994).

During a sedimentation process, the particles are pulled away from the axis of rotation by a centrifugal force. This motion is opposed by a frictional force of equal but opposite sign. When the gravitational force is generated in an ultracentrifuge, there are three forces acting on the hydrated macromolecules (Figure 1.20 A):

- A centrifugal force $F_c = \omega^2 rm$ *(ω is the angular velocity, m is the mass, and r is the distance from centre of rotation).*
- A buoyant force $F_b = - \omega^2 rm_0$ *(ω is the angular velocity, m_0 is the mass of the solution displaced by the macromolecules, and r is the distance from the centre of rotation).*
- A frictional force $F = -fn$ *(f is the frictional coefficient that is proportional to the viscosity of the solvent and the dimensions of the particle, n is the velocity of the particle relative to the centrifuge tube).*

A technique called zonal, band, or most commonly gradient centrifugation allows the separation of proteins with different sedimentation coefficients. The sedimentation coefficient S is defined as the velocity of a particle divided by the centrifugal field strength. It is calculated by using Svedberg equation (equation 2) and it is usually expressed in Svedberg units (S), which is equal to 10^{-13} s:

$$S = M (1-V_s\rho) / f \quad \textbf{(2)}$$

where M is the mass of the particle, V_s *is the partial specific volume of the particle, ρ is the density of the medium and f is the frictional coefficient (a measure of the shape of the particle)* .

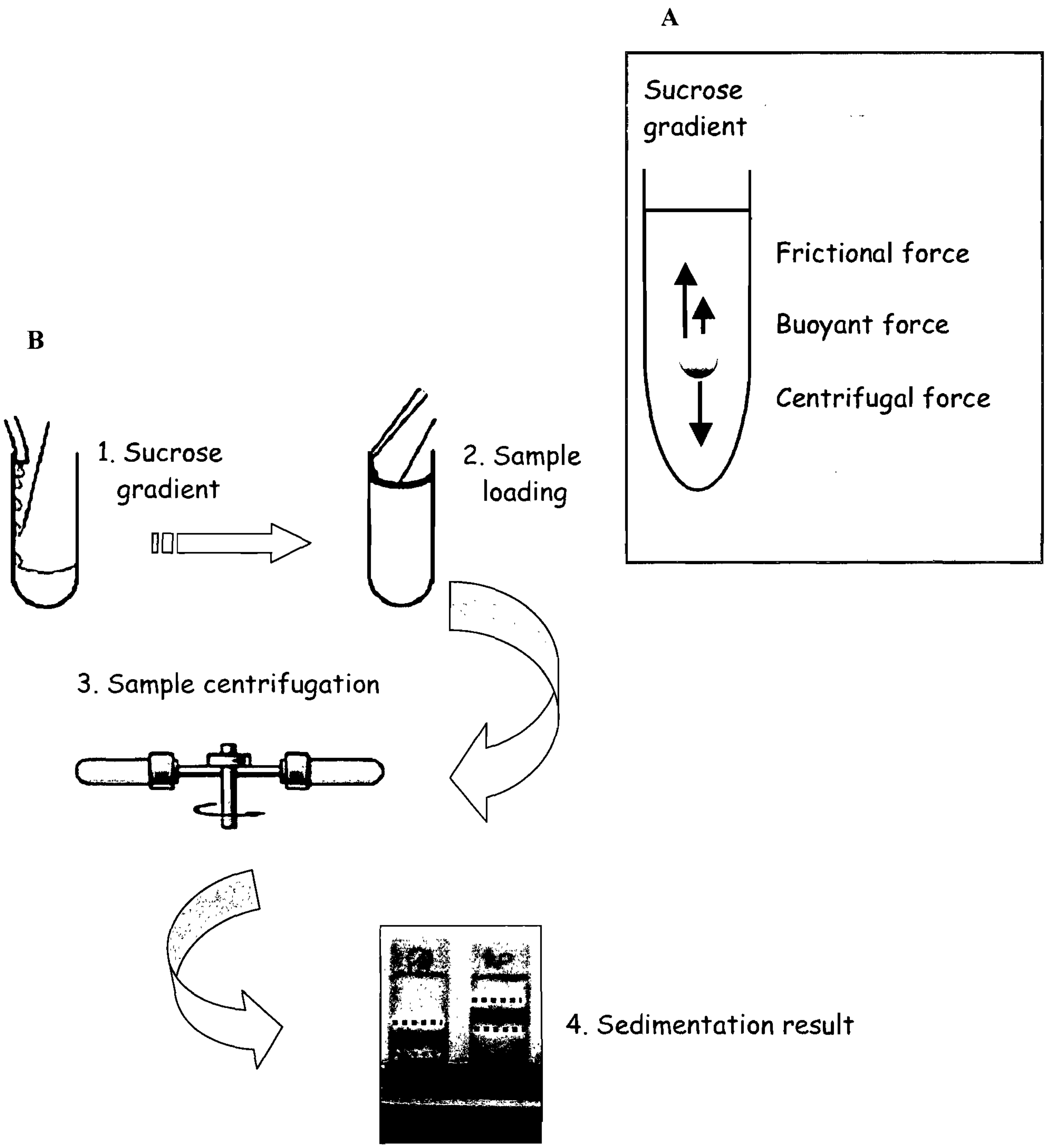


Figure 1.20: (A) Forces involved in a sedimentation experiment. (B) Velocity sedimentation experiment.

Based on equation 2, several important conclusions can be made: (i) First, the sedimentation velocity of a particle depends in part on its mass. Heavier particles sediment more rapidly than lighter ones having the same shape and density, (ii) the shape influences the sedimentation velocity since it affects the viscous drag. The frictional coefficient f of a compact particle is smaller than that of an extended particle of the same mass. Hence, elongated particles sediment more slowly than spherical ones of the same mass, (iii) dense particles move more rapidly than less dense particles, because the opposing buoyant force (Figure 1.20 A) is smaller for the denser particle, (iv) the sedimentation velocity also depends on the density of the solution (ρ) (particles sink when $\rho < 1$, float when $\rho > 1$, and do not move when $\rho = 1$).

A sucrose density gradient is created by sucrose solutions of varying concentrations overlaying solutions of lower concentrations into solutions of higher concentrations in a centrifuge tube. For example, a sucrose gradient may consist of layers extending from 20% (wt/wt) sucrose to 5% sucrose in 2% increments (the actual gradient range selected depends on sample to be purified). A sample solution containing the particles to be analysed is layered on top of the sucrose gradient (5-20%) (wt/wt). The loaded gradient is subjected to centrifugation at constant speed for a predetermined period of time. Upon centrifugation, the particles move through the gradient into separate zones depending on their size and density. The velocity of the moving particles will continually decrease as they progress into sucrose solutions of increasing concentration. If the protein solution contains a mixture of proteins of different sizes, multiple zones form after performing the ultracentrifugation for prolonged time periods. The centrifugation is discontinued before any of the separated zones reach the bottom of the tube, since the density of some zones may be higher than the highest density area in the gradient.

1.12.2. Light scattering

Unlike other techniques of characterization, light scattering (LS) does not require standards and experiments can be performed at various pH values, ionic strengths and concentrations. There exist two types of light scattering studies. Measurements of the angular distribution of time-averaged scattered intensity is known as static light scattering (SLS) and measurements of the time dependent scattered intensity or dynamic light scattering (DLS) (Berne & Pecora, 1976; Chu, 1991). According to the light scattering theory, when light interacts with matter, the electric field of the light induces an oscillating polarization of electrons in a molecule, which serve as secondary sources of light and consequently scatter light. The frequency shifts, the intensity, and the angular distribution of the scattered light are related to the size, shape and molecular interactions of the scattering molecules.

In static light scattering, one measures the intensity of the light scattered by a solution at a given time as a function of the scattering angle and concentration of the solution. In dilute solution, the relationship between the scattered intensity, the molecular weight of the macromolecule and the concentration of the solution is given by the Rayleigh-Gans-Debye or Zimm equation (equation 3):

$$\frac{K^*c}{R(\Theta)} = \frac{1}{M_w P(\Theta)} + 2A_2c \qquad \textbf{(3)}$$

where,

$R(\Theta)$ is the excess intensity of the scattered light at given angle (Θ)

c is the sample concentration.

M_w is the weight-average molecular weight.

A_2 is the second virial coefficient.

K^* is an optical parameter.

$P(\Theta)$ is the particle scattering function that depends on the particle shape and describes the angular dependence of scattered light.

Three important parameters can be derived from a light scattering measurement: (i) the weight-average molecular weight of macromolecules, (ii) the radius of gyration $<r_g^2>$, which is a parameter that characterizes the size of particle of any shape. For a particle consisting of different mass m_i, each located at a distance r_i from the centre of mass, the radius of gyration, is defined as the square root of the mass-average for all the mass elements, and (iii) the second virial coefficient, which is a property that describes the interaction strength between the particles and the solvent or other dispersant medium ($A_2>0$ means that the solvent is good and the particles tends to remain stable in solution, $A_2<0$ means that the solvent is not good and therefore the particles may aggregate, $A_2=0$ means that the particle-solvent interaction strength is equivalent to the molecule-molecule interaction strength, the solvent is then called a theta solvent).

Dynamic light scattering (DLS) measurements consist in determining the time dependence of the light scattered from a very small region of a solution over time ranging from tenths of a microsecond to milliseconds. If the light is coherent and monochromatic, as the light of a laser beam, it is possible to observe the time-dependent fluctuations of the scattered intensity, with a detector such as a photomultiplier tube capable of operating in the photon counting mode. Analysis of the time dependence of the intensity fluctuation yields the diffusion coefficient of the particles in and out of the region being studied (Brownian motion) from which, via the Stokes Einstein equation, knowing the viscosity of the medium, one calculates the hydrodynamic radius (R_h) of the particles in solution. R_h is the radius of a hypothetical hard sphere that has the same diffusion rate as the particle under examination (Equation 4).

$$R_h = \frac{KT}{6\pi\eta D} \qquad \mathbf{(4)}$$

R_h is the hydrodynamic radius

k is Boltzmann's constant.

T is the temperature in degree Kelvin.

D is the diffusion coefficient.

η is the solvent viscosity.

DLS and SLS are often used for the characterization of proteins and their aggregates, the precrystallization processes and the structures of protein-surfactant complexes. As an example, the molecular weight and radius of gyration of many proteins in solution have been determined by measuring the angular dependence of scattered light (Cantu *et al.*, 1991; Pusey, 1991; Gustke *et al.*, 1994; Shen *et al.*, 1994; Velev *et al.*, 1998; Rathore *et al.*, 2000). The hydrodynamic radius and size distribution of proteins in solutions were measured by dynamic light scattering (Hamano *et al.*, 1991; Sluzky *et al.*, 1991; Chu *et al.*, 1995; Xu & Smart, 1996; Bell *et al.*, 1997; Asherie *et al.*, 1998; Zubrzycki *et al.*, 1998; Fabain *et al.*, 1999). The two techniques have also been applied to monitoring protein aggregation and dissociation, since these phenomenons are very sensitive to changes in the size of molecules (Doty *et al.*, 1952; Kadima *et al.*, 1993; Beretta *et al.*, 1997; Schaper *et al.*, 1997; Judge *et al.*, 1999). The aggregation behaviour of proteins is a crucial phenomenon because it affects their biological activity and their ability to form crystals (Muschol & Risenberger, 1996; Rosenberger *et al.*, 1996; Neal *et al.*, 1999), which is extremely important for the structure analysis of proteins. Light scattering techniques give also access to the second virial coefficient, which is a measure of macromolecular interactions to solvent and can be used to predict the crystallization properties of a sample. George and Wilson demonstrated that many proteins crystallize in conditions where the second virial conditions becomes slightly negative, indicating net attractive interactions between proteins (George & Wilson, 1994). These are of particular important for the biopharmaceutical industry as developing new strategies for modulating such interactions, which can lead to the discovery of new therapeutic compounds.

1.12.3. Capillary electrophoresis (CE)

The process of electrophoresis is defined as the differential movement or migration of ions in an electric field. A positive electrode (anode) and a negative (cathode) electrode are placed in a solution containing ions. When a voltage is applied across the electrodes, solute ions of different charges will move through towards the electrode of opposite charge. The separation by electrophoresis relies on differences in the speed of migration of ions or solutes. The speed or velocity of ions migration is given by equation 5:

$$\upsilon = \mu_e . E \qquad \textbf{(5)}$$

υ is the ion migration velocity (ms^{-1}).

μ_e is the electrophoretic mobility ($m^2V^{-1}s^{-1}$), which indicates how fast a given ion can move through a given medium ($\mu_e = q/6\pi\eta r$, *where q is the charge of the ion,* η *is the solution viscosity and r is the ion radius*).

E is the electric field strength (Vm^{-1}).

From the electrophoretic mobility equation, one can conclude that differences in charge-to-size ratio of analyte ions result in a difference in the electrophoretic mobility. Higher charge and smaller size confer high mobility, whereas lower charge and larger size confer lower mobility. This is in fact very important because different ions and solutes will have different electrophoretic mobility and so different migration velocity at the same electric field strength. These differences in electrophoretic mobility make it possible to separate mixtures of different ions and solutes.

Capillary electrophoresis (CE) includes several separation techniques that use narrow silica capillaries. It was designed to separate species based on their size to charge ratio in the interior of the capillary filled with an electrolyte under the influence of an electric field. It has developed rapidly since the early 1980's (Jorgenson & Lukas, 1983; Hjerten, 1985; Karger, 1990; Kuhr & Monnig, 1992). It is a simple system with a relatively low surface-to-volume ratio, an extremely large separation power, reasonable speed and good automation prospects. It allows high resolution separation (with theoretical plate numbers often exceeding several hundred thousand) and reproducible quantification.

Another method, frontal analysis continuous capillary electrophoresis (FACCE), was developed. FACEE involves continuous sampling, integrating sample injection and separation into one process. The separation profile in FACCE is a discrete and progressive plateaus and it allows the determination of free and bound ligands. It was used for substrate-ligand binding studies, such as protein association with drugs (Chu & Whitesides, 1992; Kraak *et al.*, 1992; Ohara *et al.*, 1995; Oravcova *et al.*, 1996), with inorganic ions (Heegaard & Robey, 1993), with sugars (Honda *et al.*, 1992), with micelles (Sasa & Takeda, 1993; Karim *et al.*, 1994), and with polyelectrolytes (Gao *et al.*, 1997). It has also been used to investigate the binding of surfactant onto amphipols (Diab *et al.*, 2007). The separation profile obtained in FACCE is suitable for binding studies, in which the amount of free or bound ligands as well as the binding constant can be calculated. The accuracy and reproducibility rely on the correct selection of the type of capillary and the conditions of the analysis. As an example, the walls of the capillary used should have minimum interaction with solute in order to avoid adsorption and to ensure that the surface conditions are identical at the start of each run.

1.12.4. Isothermal titration microcalorimetry (ITC)

Physical and chemical processes are associated with heat change. When substances bind to each other, heat is either generated or absorbed. Measurement of this heat allows accurate determination of thermodynamic parameters, including binding constants (K_B), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS), providing in a single experiment a complete thermodynamic profile of the molecular interaction. Isothermal titration calorimetry gives experimental access to heat and full determination of all these thermodynamic parameters.

An isothermal calorimeter is composed of two cells (reference and sample, volume ~1.45 ml) made of a thermally conducting material, surrounded by an adiabatic jacket (Figure 1.21 A). Temperature differences between the reference cell (filled with buffer or water) and the sample cell containing the sample solution are detected using thermocouple circuits. A constant power is applied to the reference cell directing a feedback circuit that activates a heater located on the sample cell.

The titration involves the addition at constant temperature of aliquots of known volume of a solution of titrant (ex: surfactant) from a syringe into the sample cell containing the other component (ex: polymer). Addition of titrant is automated with a highly precise syringe, which is stirred at desired speed by a computer-controlled stepper motor.

The experiment is devised in a way such that the binding sites are in large excess at the beginning of the titration. As the reaction progresses and more of the titrant are added, the binding sites available in the sample cell decrease until no net binding occurs. After each aliquot addition, the heat released or absorbed in the sample cell is measured with respect to the reference cell filled with buffer. The heat change consists of the time-dependent input of power (μcal/s) needed to maintain the same temperature between the sample cell and the reference cell. For example, in an exothermic reaction, the temperature increases, so the feedback power to the sample cell decreases in order to keep an equal temperature between the two cells. Whereas in an endothermic reaction, the opposite happens and the feedback circuit increases the power in order to keep the two cells at constant temperature. Figure 1.21 B shows the raw data and the integrated heat of an ITC experiment. Raw data represents the heat flow (power) corresponding to each injection. Integration of this power signal with respect to time yields the heat change per injection. The heat profile as a function of molar ratio of the two species can be analyzed to give the thermodynamic parameters of the interaction under investigation. Since no binding occurs once the saturation is reached, the stoichiometry and the heat change can be accurately determined.

A

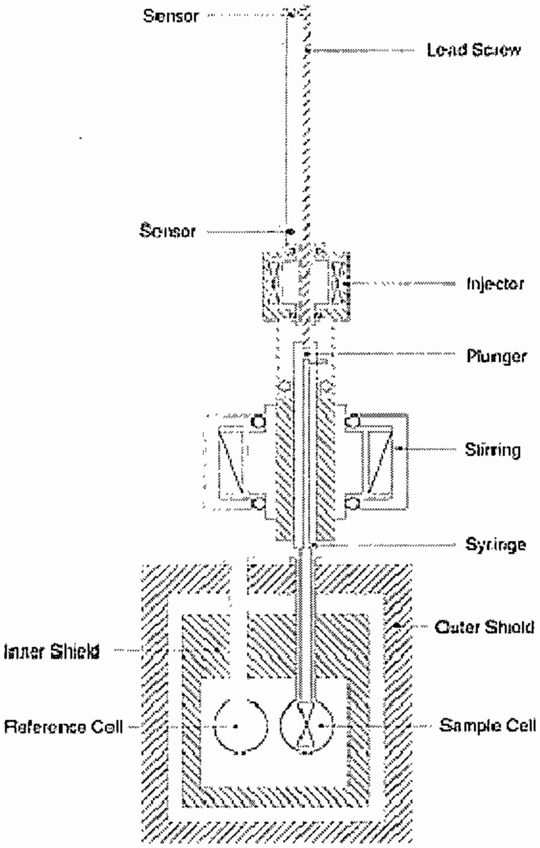


Figure 1.21: (A) Representation of ITC cells and syringe. (B) Representation of ITC titration data. This figure is taken from www.microcalorimetry.com.

B

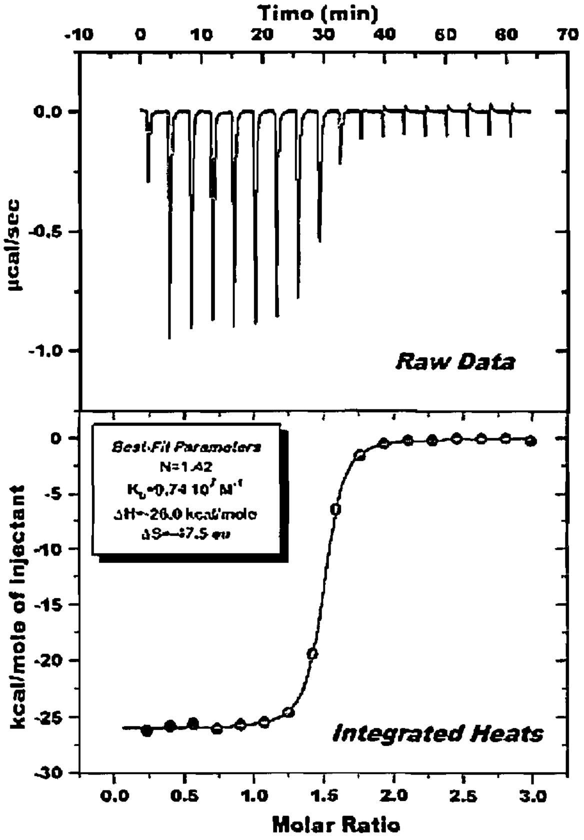


Figure 1.21: (B) Representation of ITC titration data. This figure is taken from www.microcalorimetry.com.

Also, the binding constant can be estimated by fitting the isotherm using experimental and simulated binding isotherm model (Henriques *et al.*, 2000). There exist a large number of reviews describing the principle of ITC, experimental design and various applications (Wiseman *et al.*, 1989; Ladbury, 1995; Ladbury & chowdhry, 1996; Jelisarov & Bosshard, 1999).

ITC is an essential tool in biology research and in the area of pharmaceutical development. It is the method of choice for non-destructive analysis of biomolecular stability, protein folding (Liang *et al.*, 2003), protein/ ligand interaction (Andersen *et al.*, 2003), proteins/proteins interaction (Filfil & Chalikian, 2003), drug/biomolecules interactions (such as enzyme, DNA, proteins) (Barbosa *et al.*, 2003), enzyme kinetics (Bianconi, 2003), micelles formation (Taboada *et al.*, 2003), surfactant/polymers interactions (Li *et al.*, 2003; Miyazawa & Winnik, 2003) and many other studies. Given the wide range of its applications, its ability to detect weak interactions and the ease of its use, this method forms an exciting field that is likely to undergo further development and advances in the coming years.

1.13. Binding isotherms employed in this thesis

One of the important applications of the techniques described earlier is the analysis of molecular binding. They provide quantitative information on the amount of titrant bound to its target and on the nature of the binding. The data collected allow one to determine the binding isotherms, which represent the dependence of the concentration of bound titrant to the total or free concentration of the titrant in solution. The binding isotherm gives access to binding constants, stoichiometry, and the free energy of binding.

Binding isotherms has been used widely in biochemical and pharmaceutical research to determine the thermodynamics parameters of interaction of small molecules with macromolecules and with specific receptor sites on surface of supramolecular structure (Koshland *et al.*, 1966; Linse & chazin, 1995; Saboury, 2006), and to study mixtures of surfactant (to measure monomer concentrations, micelles composition, cooperativity coefficient...) (Couderc *et al.*, 2001; Peyre, 2002). There are several approaches for the analysis of such binding isotherms (Hill, 1910; Klotz & Hunston, 1971). Among these approaches, Hill isotherm and the regular mixing theory are the most commonly used to analyze the obtained binding data.

A-Hill isotherm

Cooperative binding requires that the macromolecule contains multiple binding sites. A macromolecule exhibits cooperative binding if the affinity for the ligand changes with the amount of ligand already bound. A frequently used measure for the extent of cooperativity is the Hill coefficient, obtained by fitting the Hill equation (Equation 6) to binding data. It describes the fraction of the macromolecule saturated by ligand as function of ligand concentration.

$$\Theta = \frac{[L]^n}{K_d{}^n + [L]^n} = \frac{[L]^n}{(K_A)^n + [L]^n} \quad \textbf{(6)}$$

Θ is the fraction of ligand binding sites occupied

$[L]$ is the ligand concentration

K_d is the dissociation constant

K_A is ligand concentration producing half occupation

n is the Hill coefficient (cooperativity index)

For systems exhibiting a positive cooperativity (n >1), a ligand bound to one site increases the affinity of the substrate towards a ligand at another site (Wyman, 1964; Weber & Anderson, 1965; Dahlquist, 1978). This allows us to estimate the number of binding sites on a macromolecule and to compare it with direct physical measurements. A negative cooperativity (n < 1) interfers with the binding to additional identical binding sites and decreases the affinity of ligand for another site, (Conway & Koshland, 1968; Levitzki & Koshland 1969; Levitzki & Koshland, 1976; Bardsley & Wyman, 1978; Acerenza & Mizraji, 1997). This situation is not readily achieved. Non-cooperative binding (n =1) occurs when the ligand binds at each site independently. In other word, the Hill isotherm model introduced in 1910 (Hill, 1910) provides a simple approach to evaluate the cooperativity coefficient and gives a direct quantitative measure of how the binding of a ligand at one site affects the affinity of the other sites for the same ligand after one site is occupied.

B-Regular mixing

It allows the calculation of the free energy of micellization and the prediction of the surfactant mixture properties including the cmc, micellar composition and number of aggregation for different surfactant mixtures. In its simple version, the regular mixing is given by equation 7:

$$[\text{surfactant}]_{\text{free}} = AX_s e^{\beta(1-X_s)^2} \qquad \mathbf{(7)}$$

X_s is the molar fraction of surfactant in mixed micelles

β is an interaction parameter that represents the excess molar enthalpy contribution of mixing the micelles of two components.

A corresponds to the equilibrium constant of transfer of one surfactant from water into the micelles of the other surfactant.

The regular mixing model has been applied effectively to assess the interaction in mixtures of surfactants or polymers and surfactants in order to assess the complete thermodynamic profile of the system (Couderc *et al.*, 2001; Peyre, 2002). It has been applied successfully to describe the nonideal behavior of a number of binary surfactant systems and was shown to model accurately the critical micelle concentrations (CMC) values (Barzykin & Almgren, 1996) and the monomer-micelle equilibrium in surfactants exhibiting negative deviations from ideality (Eriksson *et al.*, 1985). This model is used in this study to analyse the binding energy and the binding isotherms between free detergent and amphipols-bound detergent. It allows us to predict the detergent concentration in equilibrium with amphipols/detergent complexes, and the heat evolved upon the transfer of detergent from water into a mixed surfactant/amphipol complex.

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CHAPTER TWO

RESEARCH PAPER

Complexation of Integral Membrane Proteins by Phosphorylcholine-Based Amphipols

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2.1. Abstract

Amphiphilic macromolecules, known as amphipols, have emerged as promising candidates to replace conventional detergents for handling integral membrane proteins in water due to the enhanced stability of protein/amphipol complexes as compared to protein/detergent complexes. The limited portfolio of amphipols currently available prompted us to develop amphipols bearing phosphorylcholine-based units (PC). Unlike carboxylated polymers, PC-amphipols remain soluble in aqueous media under conditions of low pH, high salt concentration, or in the presence of divalent ions. The solubilizing properties of four PC-amphipols were assessed in the case of two membrane proteins, cytochrome b_6f and bacteriorhodopsin. The protein/PC-amphipol complexes had a low dispersity in size, as determined by rate zonal ultracentrifugation. Short PC-amphipols ($<M> \approx 22$ kDa) of low dispersity in length, containing ~30 mol% octyl side groups, ~35 mol% PC-groups, and ~35 mol% isopropyl side groups, appeared best suited to form stable complexes, preserving the native state of BR over periods of several days. BR/PC-amphipol complexes remained soluble in aqueous media at pH ≥ 5, as well as in the presence of 1 M NaCl or 12 mM calcium ions. Results from isothermal titration calorimetry indicated that the energetics of the conversion of BR/detergent complexes into BR/amphipol complexes are similar for PC-amphipols and carboxylated amphiphols.

2.2. Keywords

Amphipols; Integral membrane proteins; Phosphorylcholine; ITC; Amphiphilic macromolecules.

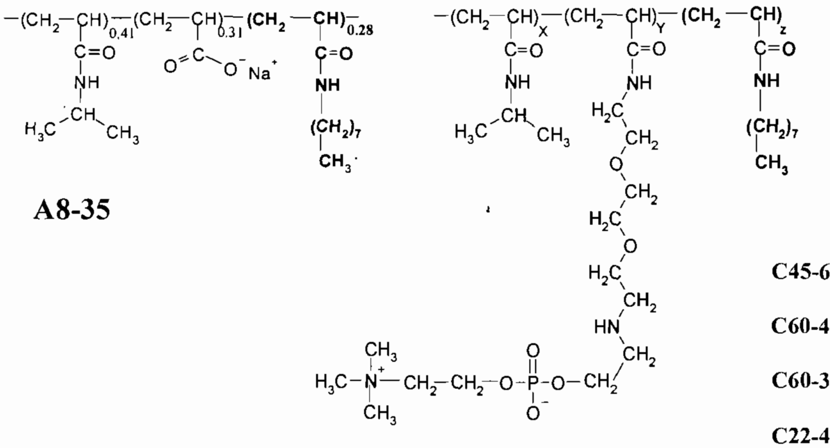
2.3. Introduction

The isolation of integral membrane proteins (IMPs) and their solubilization in aqueous media remain major stumbling blocks hampering the structural characterization of this important class of proteins and the resulting biomedical applications. A variety of detergents and polymers have been employed successfully to extract IMPs from biological membranes or to keep them soluble in water. Examples of such compounds include neutral detergents, such as surfactants of the *n*-alkyloside and *n*-alkyl(polyethylene glycol) families (le Maire *et al.*, 2000; Bowie, 2001; Garavito & Ferguson-Miller, 2001) and polymeric surfactants, such as the amphipols (amphiphilic polymers) (Tribet *et al.*, 1996; Gohon & Popot, 2003; Sanders *et al.*, 2004) and the lipopeptides (peptidic detergents) (McGregor *et al.*, 2003). Each of them however presents some drawback. IMP-adsorbed low molecular weight amphiphiles are in rapid equilibrium with the solution, which results in protein aggregation upon dilution below the detergent critical micellar concentration (cmc), and may affect IMP stability upon changes in buffer and salinity conditions. A number of the shortcomings of low molar mass detergents are alleviated through the use of amphipols, since multiple non-polar groups of a single amphipol molecule are involved in IMP complexation, which results in slower spontaneous desorption and higher affinity as compared to classical detergents

(Tribet *et al.*, 1996; Tribet *et al.*, 1997; Popot *et al.*, 2003; Zoonens *et al.*, 2007). Amphipols associate with the transmembrane domain of IMPs (Zoonens *et al.*, 2005), presumably in a way such that the hydrophobic groups of the polymer interact with the hydrophobic surface of the protein while the polar groups provide a hydrophilic corona and thereby maintain the colloidal stability of the IMP/amphipol complex in surfactant-free buffer solutions. As a consequence, IMP/amphipol complexes are vulnerable to conditions that will affect the solubility of the polymer. For instance, amphipols such as A8-35 (Tribet *et al.*, 1996), which carry weak acid groups, form soluble complexes with IMPs as long as the acid groups remain ionized. However, a decrease of solution pH below a threshold value, ~6.5 for carboxylate-based polymers, triggers the aggregation of amphipols (Gohon *et al.*, 2006) and IMP/amphipol complexes (Gohon *et al.*, 2007; Zoonens *et al.*, 2007). An increase in ionic strength above a value of ~300 mM or the presence of calcium ions also affect the colloidal stability of IMP/amphipol complexes (Picard *et al.*, 2006; and unpublished data), which limits the range of conditions accessible for IMP purification, functional assays, and structural determinations, e.g. by NMR or crystallographic techniques. This situation has prompted the development of amphipols with alternative chemical structures (Prata *et al.*, 2001; Gorzelle *et al.*, 2002; Picard *et al.*, 2006; Dahmane *et al.*, 2007; Diab *et al.*, 2007).

An attractive approach to enhance the solubility of amphiphilic polymers in buffers of high salinity and to expand the pH range in which they remain useful involves replacing the carboxylate units of the first-generation amphipols by units that possess covalently linked cationic and anionic groups (betaines) (Nagy *et al.*, 2001; Kudaibergenov *et al.*, 2006). Polybetaines tend to be poorly soluble in pure water, due to the formation of intra- and interchain ionic contacts resulting in an ionically crosslinked network structure. However, they become soluble upon addition of salts. This dissolution process can be understood in terms of the electrolyte penetrating the ionic network, screening the net attractive electrostatic interactions between the polymer chains, and hence promoting solubility (Lowe & McCormick, 2002). The addition of salt thus results in chain expansion, an effect opposite to the response of typical polyelectrolytes, which contract in salt solutions and become insoluble. Several types of polymeric betaines are known (Kudaibergenov *et al.*, 2006). Among them, polymers bearing the phosphorylcholine (PC) group are ideally suited for biochemical and biomedical applications. A number of PC-containing synthetic polymers are currently available and employed successfully as coatings of biomedical devices as well as in pharmaceutical and cosmetics formulations (Nakaya & Li, 1999). The development of these materials was based on the concept of biomimicry, in which phosphorylcholine-based copolymers create a surface that mimics a biomembrane, thus reducing biological interactions (Ishihara *et al.*, 1996). Various groups have demonstrated that PC-copolymer coatings are non-thrombogenic and that they improve the biocompatibility of ocular devices and cardiovascular implants. The hypothesis that has driven the work described here is that amphipols bearing PC-groups would *(i)* provide an enlarged pH- and salt stability window, as a result of their betaine structure, and *(ii)* possibly increase the functional stability of solubilized IMPs, due to the similarity of the structure of their hydrophilic moieties to that of phosphatidylcholine. This design strategy, whereby the anionic and cationic groups are linked to the same unit of a macromolecule, differs from the methodology of Nagy *et al.*, who reported an amphipol containing anionic and cationic groups attached to distinct units, such that in solutions of neutral pH the negative units were in 50 % excess (Nagy *et al.*, 2001).

None of the PC-based copolymers currently available can be employed readily as amphipols, which, to be effective, need to have a controlled architecture and lipophilic/hydrophilic balance, as demonstrated by previous studies on carboxylate-based amphipols (Tribet *et al.*, 1996). Miyazawa and Winnik (Miyazawa & Winnik, 2002) recently devised a versatile synthesis of PC-containing amphiphilic polymers by post-modification of a copolymer of *n*-alkylacrylamide, *N*-*t*-BOC-*N*-[(ethylenedioxybis(ethyl) acrylamide] and *N*-isopropylacrylamide. Using the design rules established for amphipols of the A8-35 class, we prepared four PC-based amphipols, which are random copolymers of *n*-octylacrylamide, *N*-isopropylacrylamide, and *N*-phosphorylcholine-*N*'-ethylenedioxybis(ethyl)acrylamide (Figure 2.1 and Table 2.1) and tested their ability to keep soluble bacteriorhodopsin from *Halobacterium salinarium* (BR) and the cytochrome b_6f complex from *Chlamydomonas reinhardtii.* We have compared PC-amphipols with A8-35 at the stage of IMP trapping, which involves the exchange of detergent for amphipol. We determined the amphipol/IMP ratio required to maintain IMPs soluble and assessed the size dispersity of the complexes by rate zonal sedimentation. We measured by isothermal titration calorimetry the enthalpy of mixing IMP/detergent complexes with excess amphipol. We have also assessed the stability of the IMP/PC-amphipols



A8-35

	x	y	z
C45-68 :	0.00	0.68	0.32
C60-44 :	0.26	0.44	0.30
C60-30 :	0.33	0.30	0.37
C22-43 :	0.30	0.43	0.27

Figure 2.1: Structure of A8-35 and of PC-amphipols.

complexes upon storage in neutral aqueous buffer and in solutions of acidic pH (pH 5.0), in the absence of salt and in saline solutions. Our study points to the remarkable solubility of IMP/PC-amphipols complexes in acidic media and in solutions of high ionic strength, thus extending the range of conditions accessible to studies of amphipol-complexed IMPs.

2.4. Experimental procedures

2.4.1. Materials

Water was deionized with a Millipore Milli-Q water purification system. Anilino-naphthalene sulfonic acid (ANS), dodecyl maltoside (98%, DDM), and *n*-octylthioglucoside (98%, OTG, for ITC experiments) were purchased from Sigma Chemicals and used without further purification. An OTG sample (> 99%) purchased from Anatrace was employed for protein solubilization. The five amphipols, A8-35 (Gohon *et al.*, 2006), C22-43, C60-44, C60-30, and C45-68 (Diab *et al.*, 2007) (according to the amphipol nomenclature used in (Tribet *et al.*, 1996), A and C stand for anionic and cationic, respectively, the digit adjacent to the letters refers to the molar mass (in kg.mol^{-1}), and the second digit is the molar percent of carboxylate or phosphorylcholine residues) were synthesized as described elsewhere (Gohon *et al.*, 2006; Diab *et al.*, 2007). Briefly, they were obtained by modification of copolymers *N*-*t*-BOC-*N*-[(ethylenedioxybis(ethyl) acrylamide], *N*-isopropylacrylamide, and *N*-*n*-octylacrylamide obtained by free-radical polymerization of suitable mixtures of the three comonomers carried out in dioxane at 65-70°C for 17 h using azobis(isobutyronitrile) as initiator. The precursor polymer used to prepare C22-43 was synthesized from the same monomers and initiators, but in the presence of the chain transfer agent *S*-1-isobutyl-*S*'-(α,α'-dimethyl-α''-*N*-isopropylacetamide) trithiocarbonate in order to control the molecular weight and polydispersity index of the polymer. In all cases, the precursor polymers were purified by two precipitations from THF into hexane and dried in vacuo. The polymers were characterized by gel permeation chromatography (eluent: DMF, 40°C, TSK-gel α-M and a TSK-gel α-3000, Tosoh Biosep columns). The *N*-*t*-BOC protecting group was cleaved by treatment with trifluoroacetic acid. The deprotected polymers were purified by treatment with the ion exchange resin Dowex 2X8-400 in order to remove trifluoroacetate ions. Phosphorylcholine groups were linked to each precursor polymer by treatment with phosphorylcholine glyceraldehyde and sodium borohydride. The PC-containing polymers were purified by dialysis against water. They were isolated by freeze-drying and characterized by ^{1}H NMR spectroscopy. The molecular characteristics of the polymer are listed in Table 2.1. All other chemicals were of analytical grade.

Table 2.1. Characteristics of the polymers employed in the study.

Polymer	Composition (mol %)				M_n (g/mol)	M_w/M_n
	NIPAM	AA	PC	C_8H_{17}		
A8-35[a]	41	31	--	28	9,000	1.6
C22-43[b]	30	--	43	27	22,000	1.2
C45-68[b]	--	--	68	32	45,000	2.5
C60-44[b]	26	--	44	30	60,000	2.0
C60-30	33	--	30	37	60,000	2.0

AA: acrylic acid; NIPAM: *N*-isopropylacrylamide; PC: *N*-phosphorylcholine –*N*'-[ethylenedioxybis(ethyl) acrylamide]; C_8H_{17}: n-octylacrylamide.

[a] See refs. (Gohon *et al.*, 2004; Gohon *et al.*, 2006) (batch HAPol-1).

[b] See ref. (Diab *et al.*, 2007).

2.4.2. Purification of bacteriorhodopsin (BR) and cytochrome b_6f

Purple membrane from *H. salinarium* (strain S9) was solubilized for 48 h in 100 mM OTG in 20 mM sodium phosphate buffer (pH 6.8) (Seigneuret *et al.*, 1991). Soluble BR/OTG complexes were recovered in the supernatant after centrifugation for 30 min at 200,000 × *g* in the TLA100 rotor of a Beckman TL100 centrifuge and diluted 3-fold in buffer to obtain a 33 mM OTG stock solution; the final BR concentration was ~1 g/L, determined from the absorbance at 554 nm ($\varepsilon = 1.60$ L g^{-1} cm^{-1}) (Seigneuret *et al.*, 1991) using a UV-visible Hewlett-Packard 8453 diode array spectrophotometer.

Cytochrome b_6f complex containing a six-histidine tag at the C-terminus end of cytochrome *f* was produced by a genetically modified strain of *C. reinhardtii* (Stroebel *et al.*, 2003). The protein was purified as described by Stroebel *et al.* (Stroebel *et al.*, 2003) from thylakoid membranes solubilized with DDM, followed by anion-exchange (Source 30Q, Amersham) and immobilized nickel (HiTrap chelating, Amersham) chromatography. The final b_6f concentration in 0.2 mM DDM and 20mM Tris-HCl pH 8.5 buffer was 0.4 g/L (~4 µM cytochrome *f*), as determined from the redox difference spectra at 554 nm and 563 nm (Pierre *et al.*, 1995). After six days of storage at 4°C, the preparation comprised a mixture of the inactive monomer and the native dimer forms of b_6f, as revealed by the two bands obtained by rate zonal sedimentation in DDM solution (Figure 2.3A) (Breyton *et al.*, 1997).

2.4.3. Amphipol solution preparations

Stock polymer solutions (2 to 20 g/L) were prepared by dissolving freeze-dried samples in deionised water under gentle stirring for at least 2 h at room temperature. The polymer solution in water was supplemented with concentrated buffer (100 mM NaH_2PO_4-Na_2HPO_4, pH 6.8) and NaCl in amounts such that the final concentrations of phosphate and NaCl were 20 mM and 100 mM, respectively. The solutions were filtered through a 0.22-µm syringe filter (Millex, Millipore USA) prior to measurements.

2.4.4. Fraction of BR maintained soluble by polymers

Aliquots of polymer stock solutions were added to a stock solution of BR/OTG in 33 mM OTG (20 µL) to reach final polymer/BR ratios of 1-12 g/g. The resulting solutions were incubated for 15 min at 4°C in the dark. They were mixed with 160-180 µL of a solution of 100 mM NaCl, 20 mM NaH_2PO_4-Na_2HPO_4, pH 6.8, to reach a final OTG concentration of 3.3 mM, i.e. below its cmc (~10 mM). After a second incubation for 10-15 min., samples were centrifuged in the A-110 rotor of a Beckman Airfuge centrifuge (20 psi, ~210,000 × *g*, 10 min.). The absorbance of the initial samples and supernatants at 554 nm was measured to determine the fraction of soluble complexes. In the case of polymer C60-44, some experiments were carried out with polymer solutions that had been centrifuged (10 min, 200,000 × *g*) prior to mixing with the BR stock solution.

2.4.5. Trapping of IMPs in amphipols and rate zonal sedimentation

To obtain BR/amphipol complexes depleted of OTG, a BR/OTG stock solution (~1 g/L BR, 33 mM OTG, 20 mM phosphate buffer) was mixed with a solution of polymer in 33 mM OTG, 20 mM NaH_2PO_4-Na_2HPO_4, pH 6.8, with or without 100 mM NaCl. The final polymer/BR ratio was 9:1 g/g. After incubation for 15 min. in the dark at 4°C, the solution was treated with Bio-Beads SM-2 (Bio-Rad Bio-Beads/OTG 10:1 wt/wt) and the mixture was stirred in the dark at 4°C overnight. Bio-Beads are known to adsorb detergents but not amphipols (Zoonens *et al.*, 2007). The supernatant (100-200 μL) was layered onto 2 mL of a 5-20% (wt/wt) linear sucrose density gradient in the same buffer, except for the absence of detergent (20 mM NaH_2PO_4-Na_2HPO_4, pH 6.8, with or without 100 mM NaCl). The tubes were centrifuged at 4°C for 5 h at 55,000 rpm in a Beckman TLS 55 rotor (~250 000 × *g*), or for 18 h at 37,000 rpm in an MLS50 one (~145,000 × *g*). Typically 18-20 fractions (120 μL or 200 μl, respectively) were collected from the top of the tubes and analyzed by UV-visible absorbance spectroscopy. A control sedimentation experiment was performed simultaneously with the BR/OTG stock solution diluted in the phosphate buffer down to ~15 mM OTG (~0.5g/L BR), without removal of OTG. The BR/OTG samples were layered onto 5-20 % sucrose gradients in 20 mM phosphate buffer, 10 mM OTG, and centrifuged in the same conditions as the BR/polymer samples.

The same procedure was adopted for the sedimentation of b_6f/amphipol complexes, starting with a mixture of b_6f/DDM complexes and the polymer in a 20 mM Tris/HCl buffer, pH 8, NaCl 100 mM, in which the DDM concentration exceeded the cmc of DDM, incubated overnight with Bio-Beads to remove DDM. The concentration of b_6f in the collected fractions was determined from their absorbance at 554 nm, 420 nm, and 280 nm.

2.4.6. Rate zonal sedimentation of polymers in sucrose gradients

ANS (0.2 mM final concentration, from a 10 mM stock solution in water) was added to solutions of amphipols (1 g/L) in 20 mM NaH_2PO_4-Na_2HPO_4, pH 6.8. Aliquots (100 µL) of these solutions were layered onto a 2-mL 5-20 % (w/w) linear sucrose density gradient in the same buffer, containing 35 µM ANS. The tubes were centrifuged at 20°C (35,000-45,000 rpm, Beckman MLS 50 rotor, ~145,000-210,000 × *g*). The position of the fluorescent band was noted for increasing centrifugation times (1-18 h).

2.4.7. Isothermal titration calorimetry

Measurements were carried out with a VP-ITC instrument from Microcal Inc (USA). Prior to each experiment the sample cell (1.43 mL) was filled with a polymer solution. BR solubilized in OTG (cf. section 2.4.2) was purified by centrifugation on a 5–20% (wt/wt) sucrose gradient in 15 mM OTG, 20 mM NaH_2PO_4-Na_2HPO_4 buffer pH 6.8 (Beckman MLS50 rotor, 18 h at 37,000 rpm, ~145,000 × *g*). The band of BR/OTG was collected and dialyzed for 48 h against the same buffer supplemented with 15 mM OTG and 100 mM NaCl, to remove the sucrose and to achieve a good control of the concentrations of detergent and salt in equilibrium with the protein. The heat exchanges generated by the interactions of

BR/OTG complexes with the polymer were obtained from titrations of the BR/OTG solution (0.45 g/L BR) into aqueous polymer solutions (either 1.0 or 5.0 g/L) in the same buffer. During the titration the 300-μL syringe containing the BR/OTG solution was stirred at a speed of 300 rpm, allowing rapid mixing of the aliquots injected into the sample cell (Blandamer *et al.*, 1998). Aliquots (2 to 10 μL) were injected into the sample cell at 300-s intervals. All measurements were carried out at 25°C. Data were analyzed using the Microcal ORIGIN software. The experimental enthalpy change h_i resulting from the *i-th* BR/OTG solution injection was obtained by integration of the raw data signal. The integrated molar enthalpy change per injection (ΔH_i, in J/mol BR) was obtained by dividing h_i by the number of moles of OTG or BR added, n_i; hence $\Delta H_i = h_i/n_i$, resulting in enthalpograms, which are plots of ΔH_i as a function of free OTG or BR concentrations in the calorimeter sample cell.

2.4.8. Assessment of the stability of BR/amphipol solutions

Two fractions of purified BR/amphipol complexes were collected by sedimentation experiments (section 2.4.5, fractions containing the maximum BR concentration). The absorbance spectrum of these fractions kept in the dark at 4°C was measured regularly for 11 days. Alternatively, an aliquot of the fraction was supplemented with either 5 M NaCl (final NaCl 1 M), 250 mM citric acid-NaOH buffer, pH 5.0 (final citrate 25 mM), or $CaCl_2$ 120 mM (final Ca^{2+} 12 mM). The UV-visible spectra of the samples were measured immediately after supplementation and after centrifugation (10 min at ~200,000 × *g*) at incubation times 15 min and 4 hours. Similar measurements were carried out for b_6f/C60-44 solutions in the same buffers containing either 1 M NaCl, or 25 mM citrate buffer to check the stability and absence of aggregation after incubation for 1 hour.

2.5. Results

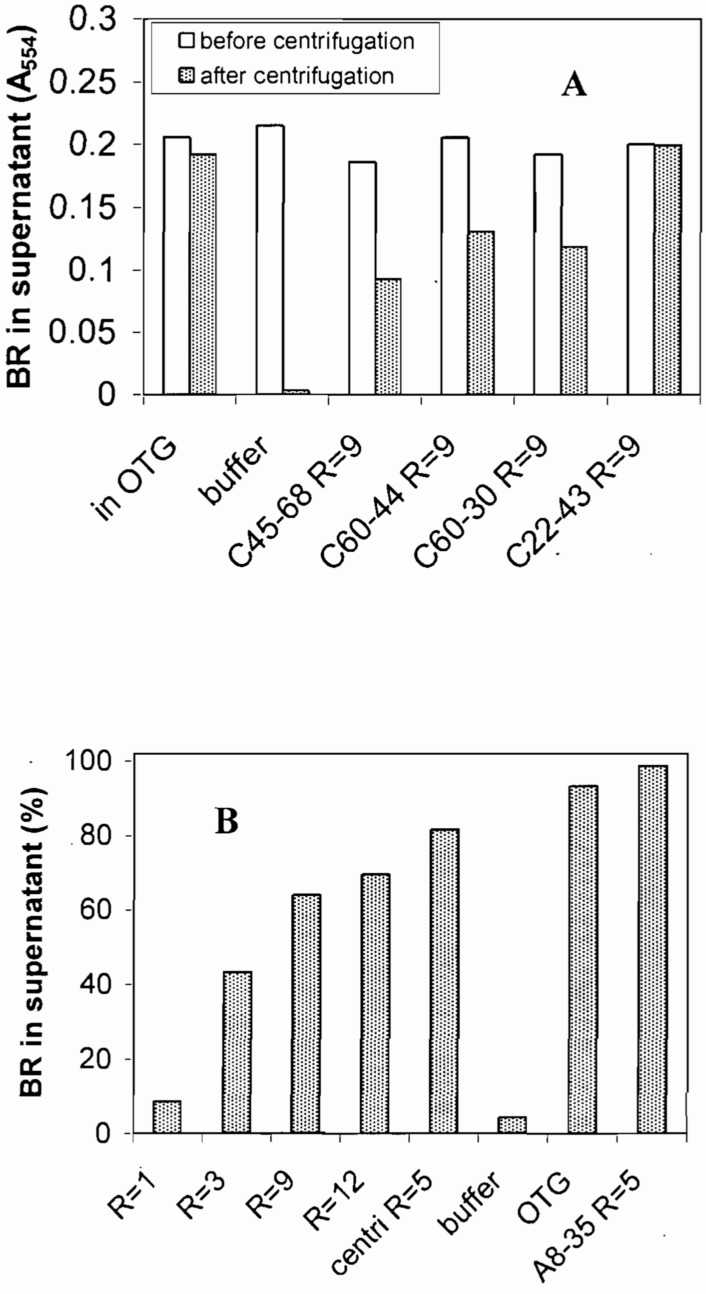
2.5.1. Design and properties of PC-amphipols

Four different PC-amphipols were used in this work (Figure 2.1 and Table 2.1). Three samples, C45-68, C60-44, and C60-30, were prepared by conventional free radical polymerization and exhibit a broad distribution of chain sizes characterized by a polydispersity index above two. To illustrate the implications of this polydispersity index, estimates of the average molar masses of truncated populations of chains help in understanding the implications of a high polydispersity value : for example, in C60-44 having an average mass of 60,000 g/mol, as determined by gel permeation chromatography, the first 10 % by weight of chains (i.e. the shortest macromolecules) have a molecular weight of 24,000 g/mol whereas the last 10 % by weight (longest macromolecules) in the total distribution have a mass of 285,000 g/mol. The chains that are present in significant amount thus cover a range of lengths of one decade. The dispersity of the C60-44 chains is similar to that of the amphipol A8-35 developed by Tribet, Audebert & Popot (Table 2.1). In contrast, the size distribution of C22-43, which was obtained by a controlled free-radical polymerization (Chiefari *et al.*, 1998), is much narrower. Estimates of the average molar masses of the 10 % shortest and 10 % longest chains in C22-43 gave 13,000 g/mol and 34,000 g/mol, respectively, which are values close to the average value (22,000 g/mol) of the mass distribution. The PC-amphipols C22-43, C60-44 and C60-30 are random copolymers of the neutral monomer *N*-isopropylacrylamide, the oleophilic monomer *n*-octylacrylamide, and a hydrophilic unit bearing a phosphorylcholine group. The fractions of the three monomers in each sample are listed in Table 2.1. We also used a PC-amphipol (C45-68) consisting exclusively of PC units and *n*-octyl units. Note that all the PC-modified polymers are cationic under the conditions of our measurements as a consequence of the presence of a secondary amine in the fragment linking the PC group to the polymer main chain (Diab *et al.*, 2007).

Previous dynamic light scattering studies indicated that, in aqueous media, PC-amphipols self-assemble into particles containing a small number of polymer chains. Thus, in the pH 6.8 phosphate buffer used in this study, C22-43 forms micelles having a Stokes radius of 4.4 ± 1.1 nm, without detectable amounts of larger particles (Diab *et al.*, 2007). C60-44 assembles into larger objects which have a hydrodynamic radius of ~15 nm and a broader size distribution, as compared to C22-43 particles. A small fraction of larger aggregates (~150 nm in size) could be detected in solutions of C60-44. These particles were effectively removed by centrifugation (10 min. at 200,000 × *g*).

2.5.2. Formation of bacteriorhodopsin/PC-amphipols complexes.

The ability of PC-amphipols to form water-soluble complexes with IMPs was examined using purified preparations of BR ($M \approx$ 27,000 g/mol) in octylthioglucoside (OTG). In a typical experiment, the protein/detergent solution was supplemented with PC-amphipols and diluted with buffer such that the concentration of OTG dropped below its cmc. The sample was then subjected to centrifugation and the extent to which the protein was kept soluble was determined from the absorbance of the supernatant (Tribet *et al.*, 1996). In the absence of amphipols, this dilution–centrifugation procedure resulted in almost complete precipitation of the protein (Figure 2.2A). In contrast, in the presence of all PC-amphipols tested, as well as with A8-35, a significant fraction of BR was detected in the supernatant as long as the polymer/BR weight ratio was higher than 3 g/g (Figure 2.2A and 2.2B). The fact that BR remained in the supernatant suggests that the polymers bind to the protein, thereby preventing its aggregation and precipitation. With C45-68, C60-44 and C60-30, a plateau of ~70% retention of the protein in the supernatant was reached for polymer/protein weight ratios above 5/1. As noted earlier (section 2.5.1), solutions of C60-44 in buffer contain a small amount of large particles, which can be removed by centrifugation. The presence of such aggregates may have decreased the retention yield of the protein. Indeed, using a pre-centrifuged C60-44 solution to prepare BR/C60-44 complexes enhanced the solubility of BR to 80% (Figure 2.2B). In all cases, the UV-visible spectrum of BR, which is highly sensitive to the functional state of the protein, did not change significantly upon association with PC-amphipols, indicating that these polymers can preserve the native state of the protein. In all cases, however, some loss of protein (~20%) took place, and this even at high polymer concentration. Two phenomena, protein denaturation and aggregation/precipitation, can concur to lower the overall yield.

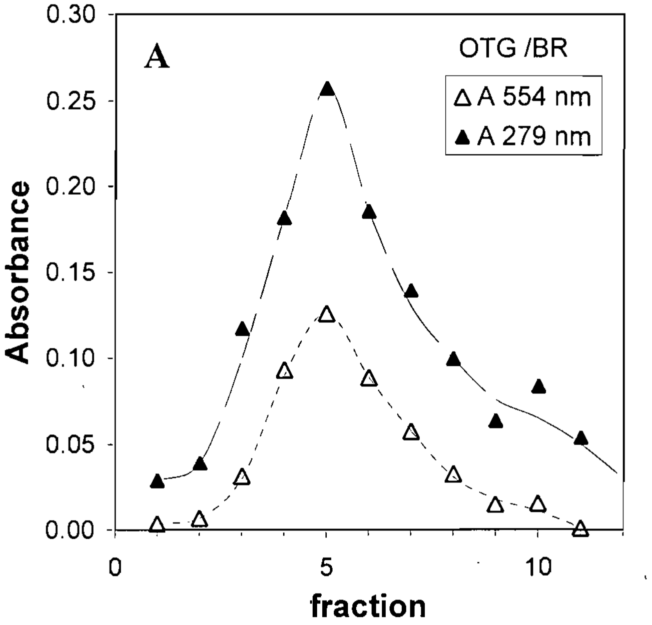


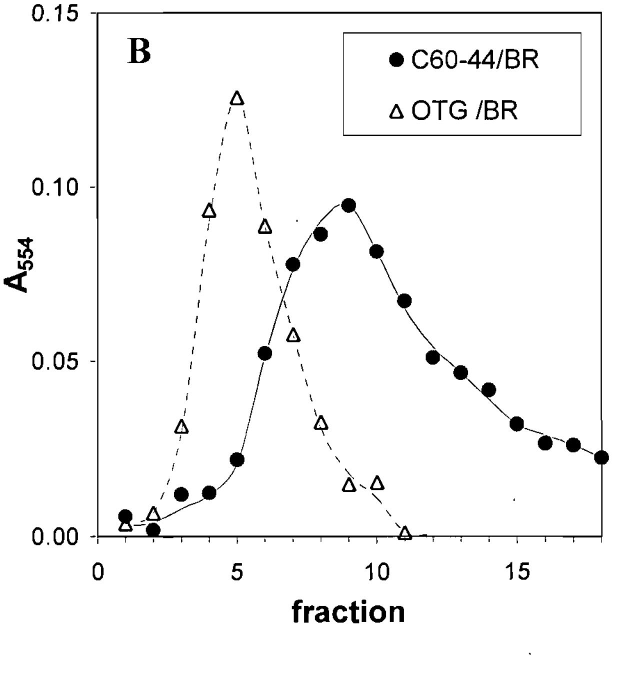
Caption on page 134

Figure 2.2: Solubility of BR at submicellar concentrations of OTG in the presence of PC-amphipols or A8-35. Aliquots of a stock solution of polymer were added to the stock solution of BR (~1 g/L BR solubilized in 33 mM OTG, 20 mM phosphate buffer, pH 6.8) to obtain various polymer/BR ratios, *R* (in g/g). After incubation for 15 min at 4°C, samples were diluted 10× in 100 mM NaCl, 20 mM phosphate buffer, pH 6.8, and centrifuged at 200,000 × *g*, 10 min., 8°C, in a Beckmann Airfuge. The fraction of BR kept soluble was determined from the absorbance at 554 nm of the supernatants. "OTG" and "Buffer" refer respectively to dilutions into the phosphate buffer, in the absence of polymer, with and without 33 mM OTG. "Centri *R* = 5" corresponds to the use of a stock solution of polymer that was pre-centrifuged (200,000 × *g*, 10 min) to remove traces of polymer aggregates prior to mixing with BR. (A) Comparison of polymers at *R* = 9; (B) solubility of BR as a function of C60-44 concentration. A polymer/BR ratio of 5 g/g corresponds approximately to mol/mol ratios of 2.2:1 (C60-44) or 15:1 (A8-35).

Denaturation is expected to increase at high ratios of APols or detergent, which may favor delipidation of the protein (the mere dilution of protein/OTG complexes into an excess of OTG results in the loss of ~10% BR). Aggregation is favored at low APol/IMP ratios which are conditions driving the formation of protein/protein interactions at the expense of protein/amphipol interactions. Milder procedures of mixing and adjustments of handling conditions (temperature, ionic strength, etc) are likely to enhance the current 80% recovery. In the present study, however, we did not attempt further optimization of the transfer from OTG.

Based on previous studies, we hypothesize that the solubility of BR in the presence of PC-amphipols is due to the formation of soluble protein/polymer complexes (Tribet *et al.*, 1996) and that the treatment with Bio-Beads (section 2.4.5) removes most of the OTG from the complexes (Gohon *et al.*, 2007; Zoonens *et al.*, 2007). We set about next to assess by rate zonal centrifugation the size dispersity of the OTG-depleted BR/PC-amphipol complexes. In Figure 2.3A, we show, as a control experiment, the absorbance at 554 nm of fractions collected after centrifuging BR/OTG complexes (i.e. the starting material for trapping experiments) in a gradient containing OTG. The sedimentation band of the OTG/BR complexes extends mainly over three consecutive fractions in the sedimentation gradient. The trailing of the band towards the bottom of the gradient indicates that, while BR is predominantly monomeric, some oligomers are also present (Gohon *et al.*, 2007). When amphipol-trapped BR was deposited onto a surfactant-free gradient, a single sedimentation band migrating towards the middle of the sucrose gradient was observed for the complexes formed with C22-43, C60-44, C60-30, and A8-35.





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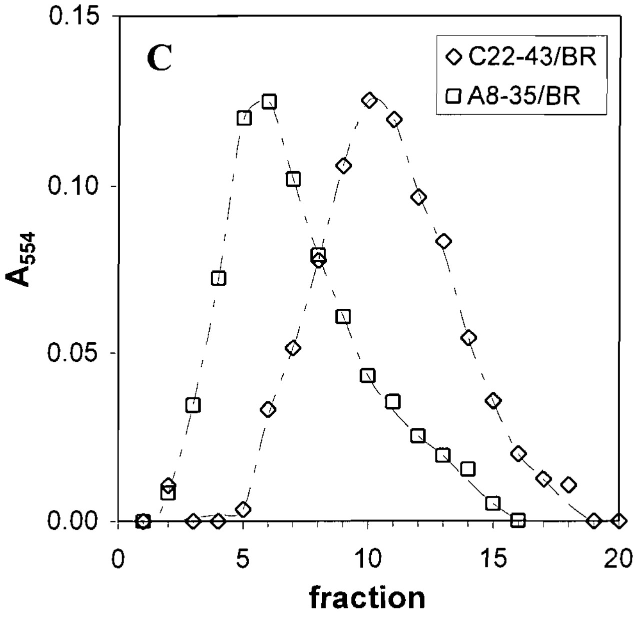

Figure 2.3: Rate zonal sedimentation of BR/OTG and BR/polymer complexes in sucrose gradients. Samples containing ~0.5 g/L BR and either a polymer/BR ratio $R = 9$ g/g, or, in the absence of polymer, ~15 mM OTG, were layered onto 5-20 % (w/w) sucrose gradients in 20 mM phosphate buffer, pH 6.8, containing (A, B) 10 mM OTG ("BR/OTG complexes"), or (B, C) no surfactant ("BR/C60-44, "BR/C22-43" and "BR/A8-35" complexes). OTG had been removed from polymer-containing samples by adsorption onto Bio-Beads prior to deposition onto the gradient (see Section 2.4). After centrifugation, 18-20 fractions were collected from the top of the tubes. The concentration of BR in the fractions was determined by absorbance measurements at (A-C) 554 nm and (A) 279 nm.

The presence of a single band confirms the absence of BR aggregation upon entrapment by any of the four polymers. In one case, however, namely that of BR/C45-68 complexes, the sedimentation band was much broader and aggregates were collected at the bottom of the sedimentation gradient (not shown). This behavior was noted only in the case of BR/C45-68 complexes. It may indicate that the chemical composition of this PC-amphipol, which comprises no *N*-isopropylacrylamide units, is not well suited to its function as an amphipol. Consequently, we did not carry out further measurements with this polymer.

Under identical conditions, BR/PC-amphipol complexes migrate deeper in the gradient than BR/OTG complexes: as shown in Figure 2.3B in the case of BR/C60-44 complexes, the protein concentration is maximal in fractions 8 and 9, instead of 4-5 for OTG/BR, which means that the polymer/BR complexes migrated twice as far during the same centrifugation time (18 h). The penetration in the gradient of BR/PC-amphipol complexes is also about twice as deep as that of BR/A8-35 complexes (Figure 2.3C). In all cases, the band containing the BR/polymer complexes featured a UV-visible spectrum characteristic of native BR (Figure 2.4), devoid of an absorbance maximum at 382 nm, the wavelength at which free retinal absorbs when released from denatured BR.

The rate at which a particle sediments depends on its molecular weight, hydrodynamic radius, and density. It seems unlikely that the BR/PC-amphipol complexes have a hydrodynamic radius smaller than that of compact BR/OTG complexes.

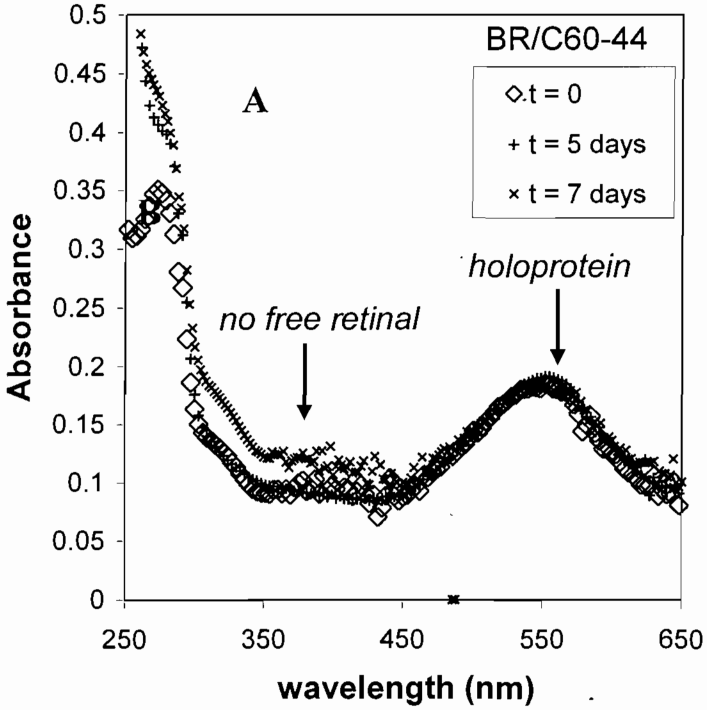

Figure 2.4: UV-visible spectra of BR/PC amphipol complexes collected from sucrose gradients and incubated in the dark at 4°C for the time indicated. (A) BR/C60-44 with 1 M NaCl added at time zero. Arrows indicate peaks corresponding to native BR (~554 nm) and free retinal (~382 nm).

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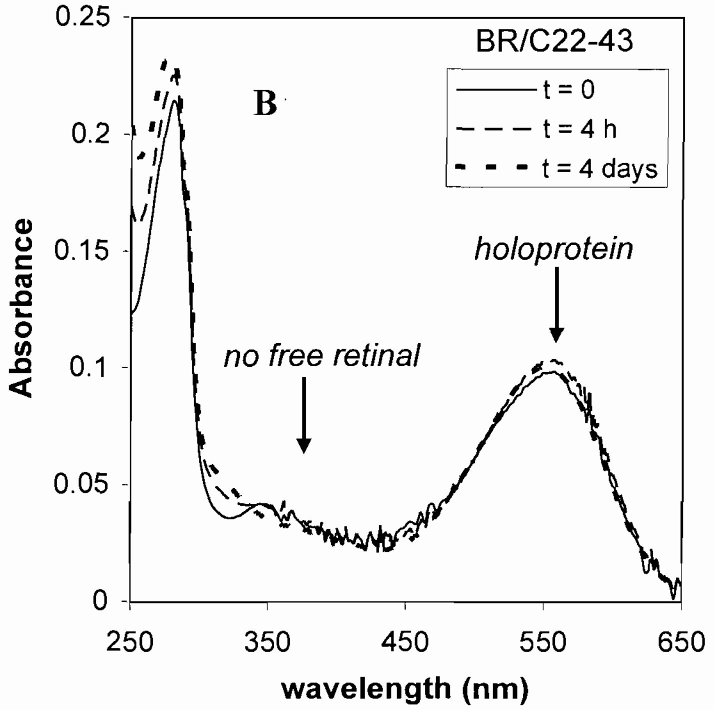

Figure 2.4: (B) BR/C22-43 with 1 M NaCl added at time zero. Arrows indicate peaks corresponding to native BR (~554 nm) and free retinal (~382 nm).

Consequently, their faster sedimentation rate points to a significantly higher density and/or larger molar mass of the particles, likely due to a higher density and/or mass of the BR-bound amphipol. In order to examine whether the polymers associated to BR have significantly different sedimentation properties, which could affect the sedimentation of the complexes, we compared the sedimentation rates of the free polymers. The sedimentation band of the polymers, in the absence of BR, was visualized with a fluorescence probe (ANS) known to partition in the hydrophobic core of surfactant micelles (Ortner *et al.*, 1979). C22-43 and C60-44, in the absence of BR, were found to sediment about twice as far as A8-35 (Figure 2.5A). The particles of A8-35 and their sedimentation properties have been extensively characterized (Gohon *et al.*, 2004; Gohon *et al.*, 2006). PC-polymer particles have similar (C22-43) or larger (C60-44) hydrodynamic radii in solution (Diab *et al.*, 2007). Consequently, in the absence of protein, the faster sedimentation rate of PC-amphipols as compared to A8-35 must arise from an increase in the density and/or the mass of the PC-amphipols particles. The sedimentation of C22-43 produces a sharp band (Figure 2.5B), whereas the band formed by C60-44 is much broader, betraying the polydispersity in size of the particles formed by this amphipol (Table 2.1). It is important to note that it is most probably because of the polydispersity in size of C60-44 particles that BR/C60-44 complexes form a significantly broader peak in the gradients than BR/OTG complexes (cf. Figure 2.3B). In the case of C22-43, which, in the absence of BR, features a much reduced particle size polydispersity, complexes with BR indeed appear nearly monodisperse in size and do not form aggregates.

Consequently, their faster sedimentation rate points to a significantly higher density and/or larger molar mass of the particles, likely due to a higher density and/or mass of the BR-bound amphipol. In order to examine whether the polymers associated to BR have significantly different sedimentation properties, which could affect the sedimentation of the complexes, we compared the sedimentation rates of the free polymers. The sedimentation band of the polymers, in the absence of BR, was visualized with a fluorescence probe (ANS) known to partition in the hydrophobic core of surfactant micelles (Ortner *et al.*, 1979). C22-43 and C60-44, in the absence of BR, were found to sediment about twice as far as A8-35 (Figure 2.5A). The particles of A8-35 and their sedimentation properties have been extensively characterized (Gohon *et al.*, 2004; Gohon *et al.*, 2006). PC-polymer particles have similar (C22-43) or larger (C60-44) hydrodynamic radii in solution (Diab *et al.*, 2007). Consequently, in the absence of protein, the faster sedimentation rate of PC-amphipols as compared to A8-35 must arise from an increase in the density and/or the mass of the PC-amphipols particles. The sedimentation of C22-43 produces a sharp band (Figure 2.5B), whereas the band formed by C60-44 is much broader, betraying the polydispersity in size of the particles formed by this amphipol (Table 2.1). It is important to note that it is most probably because of the polydispersity in size of C60-44 particles that BR/C60-44 complexes form a significantly broader peak in the gradients than BR/OTG complexes (cf. Figure 2.3B). In the case of C22-43, which, in the absence of BR, features a much reduced particle size polydispersity, complexes with BR indeed appear nearly monodisperse in size and do not form aggregates.

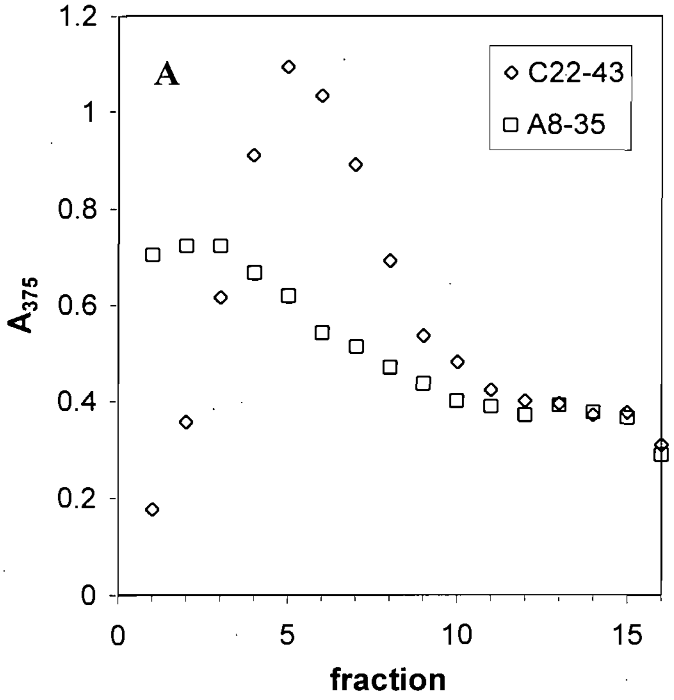

Figure 2.5: Rate zonal sedimentation analysis of polymers A8-35, C60-44 and C22-43, labeled with 200 μM ANS, in 5-20% (w/w) sucrose gradients. The gradients contained 35 μM ANS. (A) Absorbance of ANS in fractions collected from top to bottom of the gradient after an 18 h centrifugation. The same volume of samples was layered on the top of gradients (20 mM phosphate buffer, pH 6.8), which were centrifuged at 37,000 rpm (Beckman TLA centrifuge, MLS 50 rotor, ~145,000 × *g*).

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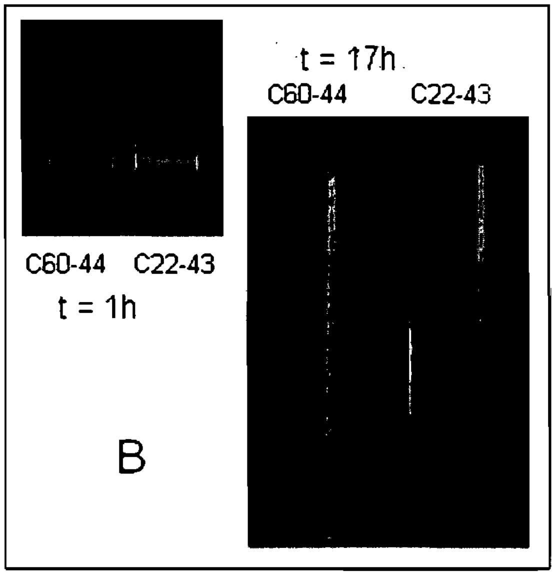

Figure 2.5: (B) Photographs of the tubes exposed to UV light after 1 h and 17 h of centrifugation. The fluorescence of ANS exposed to UV (360-400 nm) reveals the presence of polymer particles. The same volume of samples was layered on the top of gradients (20 mM phosphate buffer, pH 6.8), which were centrifuged at 37,000 rpm (Beckman TLA centrifuge, MLS 50 rotor, ~145,000 × *g*).

Absence of aggregates and a sharp sedimentation band of BR/A8-35 complexes were essential criteria in the assessment of the efficiency of A8-35 as a suitable amphiphile for the handling of membrane proteins (Tribet *et al.*, 1996; Gohon *et al.*, 2007). The only difference of behavior between the two types of BR/amphipol complexes is a downshift by four fractions for C22-43 complexes, as compared to BR/A8-35 ones, under identical centrifugation conditions (Figure 2.3C). As already noted for A8-35 (see e.g. refs. (Picard *et al.*, 2006; Gohon *et al.*, 2007; Zoonens *et al.*, 2007), these observations point to the important impact of the properties of amphipols in solution on the characteristics of their complexes with IMPs.

The contribution of the molecular properties of the polymer to the sedimentation properties of IMP/amphipol complexes should be less pronounced when the protein is significantly more massive than the polymer. To test the effect of protein molar mass on the dispersity of IMP/PC-amphipol complexes, we transferred to C60-44 or C60-30 a preparation of detergent-solubilized cytochrome b_6f. The protein samples used, which had been obtained by incubating for ~6 days at 4°C a preparation of dimeric b_6f purified in DDM (Stroebel *et al.*, 2003), contained both native b_6f dimers (16 protein subunits, $M \approx$ 215,000 g/mol) and some inactive monomers (6 subunits, 87,000 g/mol) (see refs. (Breyton *et al.*, 1997; Stroebel *et al.*, 2003)). The complexes were subjected to rate zonal sedimentation experiments similar to those carried out with BR, and fractions of the gradients were collected to measure their absorbance at 420 nm (Soret absorption band of the b_6f hemes). The mixture of monomer and dimer enables one to check on the resolution achieved (observation of two peaks in the gradients).

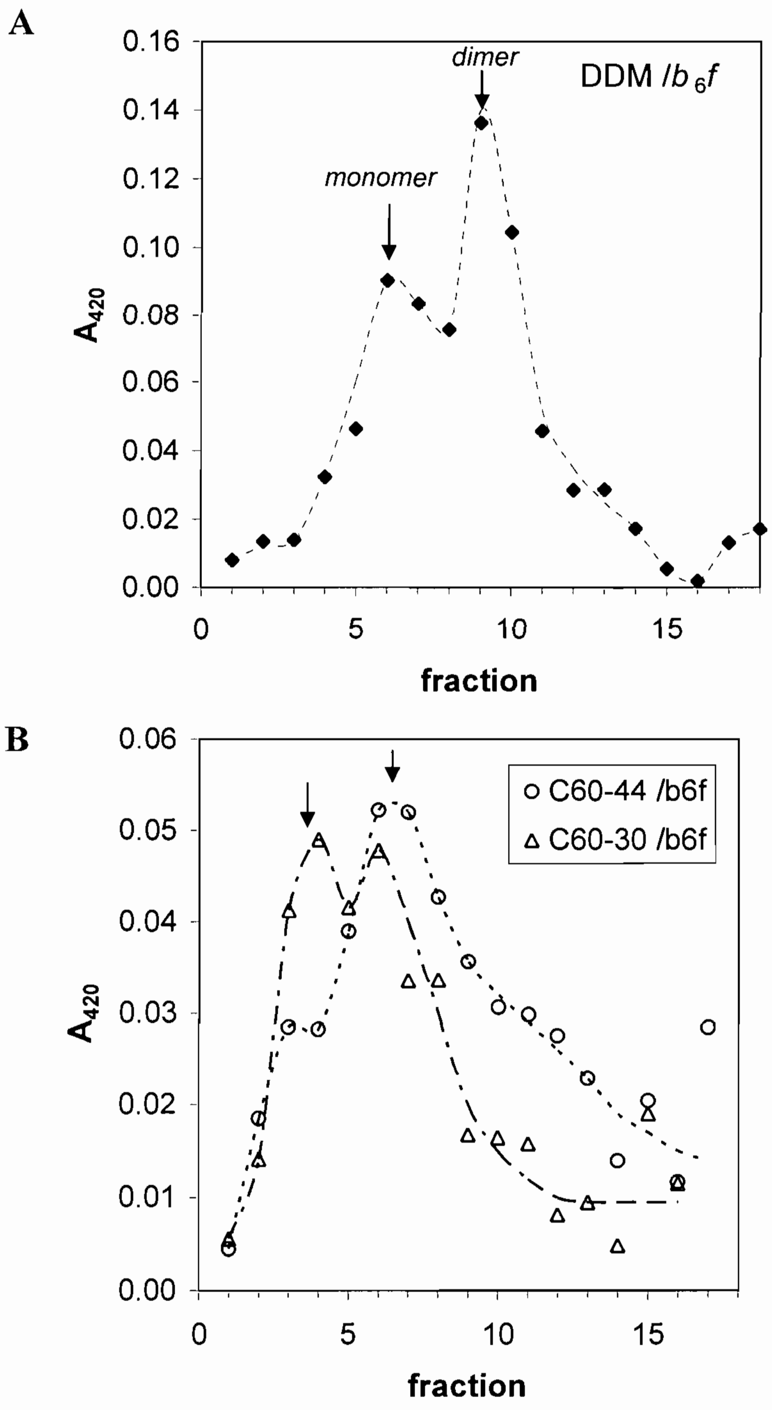

Figure 2.6: Rate zonal sedimentation of b_6f/DDM (A) and b_6f/PC-amphipol complexes (B) in sucrose gradients. The distribution of the protein was determined from heme absorbance at 420 nm.

In Figure 2.6A, we present the results of a control experiment carried out with the same b_6f sample, kept in DDM and centrifuged in DDM-containing gradients. The presence of monomer and dimer was evidenced by peaks in fractions 6 and 9, respectively. For the protein trapped by polymers (Figure 2.6B), the monomers and dimers were not as clearly distinguished as with b_6f/DDM complexes, presumably because of the broader size distribution of the complexes (arrows in the figure tentatively point to their expected positions). The b_6f/PC-amphipol complexes were recovered in fractions located at a similar depth in the gradient as b_6f/DDM complexes (namely between fractions 4 and 7, Figure 2.6B), at variance with the different rates of migration observed for the complexes formed with BR. This result indicates that the sedimentation of proteins of high molar masses is much less sensitive to the properties of the polymers entrapping them.

2.5.3. Isothermal calorimetry titrations

The energetics of the formation of IMP/PC-amphipol complexes were assessed by isothermal calorimetric titrations monitoring the heat exchange that takes place upon titration of BR/OTG complexes into a solution of amphipol. Heat is released during the transfer of a protein from a detergent solution to an amphipol solution as a result of several processes: *(i)* dispersion of the OTG micelles into monomers; *(ii)* association of OTG with the polymer; and *(iii)* formation of BR/polymer complexes. BR/OTG complexes were prepared and purified by sedimentation through a gradient containing OTG micelles (13.5 mM OTG). The resulting OTG/BR solution was equilibrated by dialysis against a solution of

13.5 mM OTG in buffer (100 mM NaCl, 20 mM NaH_2PO_4-Na_2HPO_4, pH 6.8). The polymer present in the calorimetric cell, either C22-43 or A8-35, was dissolved in the same buffer but without OTG.

Control titrations including *(i)* the titration of OTG into either buffer or a polymer solution in the same buffer and *(ii)* the titration of a buffer solution (100 mM NaCl) into a buffer of slightly different salt concentration (95 mM NaCl) are presented as supporting information (Appendix A), together with the raw data corresponding to the enthalpograms presented in Figure 2.7. Figure 2.7A shows that dilution of 13.5 mM OTG into buffer is an exothermic process (Diab *et al.*, 2007). The demicellization enthalpy of OTG has been determined to be 5.4 kJ/mol (Diab *et al.*, 2007). Upon dilution of OTG micelles into a solution of either A8-35 or C22-43, concomitant re-association of surfactant molecules to the polymer takes place. The corresponding endothermic contribution has been analyzed in detail elsewhere (Diab *et al.*, 2007). The heat evolved upon dilution of BR/OTG complexes into a polymer solution was significantly higher than the heat evolved upon addition of micellar OTG containing no BR (Figure 2.7A), implying that a large exothermic effect is associated with the overall interaction of BR/OTG with polymers. The magnitude of this effect can be established by subtracting from the signal measured upon dilution of BR/OTG complexes into polymer the contribution due to the dilution of the free OTG into the polymer (determined using the OTG solution against which the complexes had been dialysed). The enthalpy difference associated with the trapping of BR into polymers is presented in Figure 2.7B as a function of protein concentration in the ITC cell containing either A8-35 or C22-43.

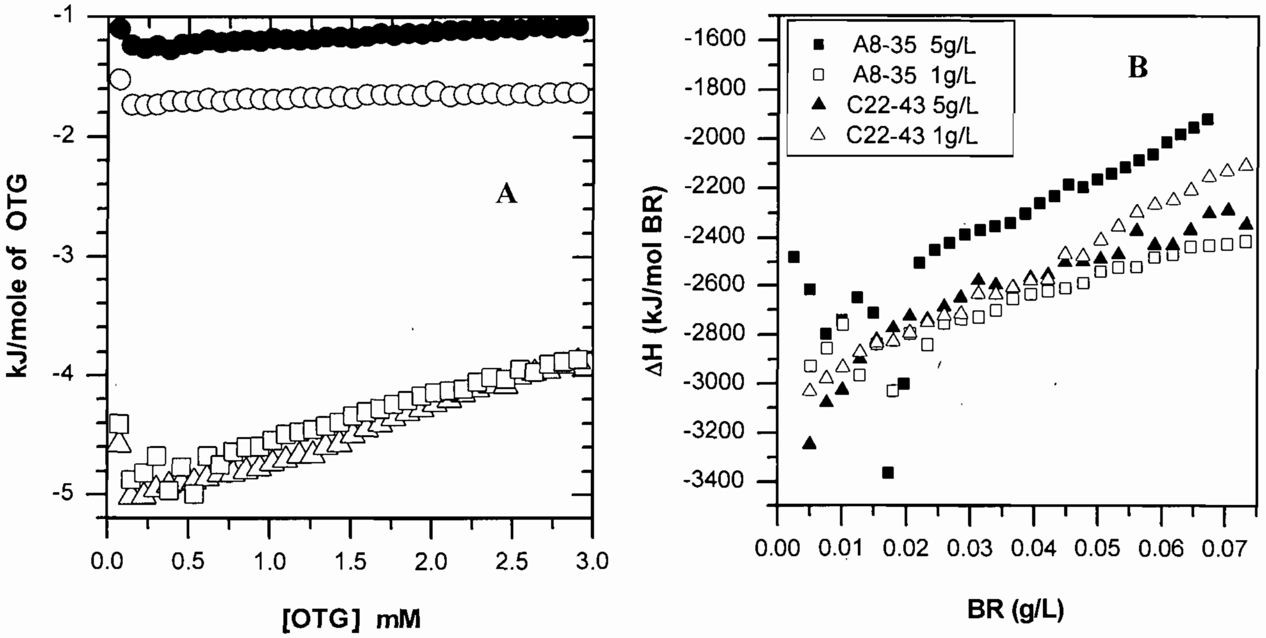

Figure 2.7: Enthalpy of dilution (Δ*H*) of OTG or BR/OTG complexes into solutions of amphipols. All solutions were prepared in 100 mM NaCl, 20 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 6.8. (A) Injection of OTG into solutions of 1 g/L C22-43 (○) or A8-35 (●), and corresponding injection of BR/OTG complexes (BR 0.45 g/L in 15 mM OTG) into 1 g/L C22-43 (Δ) or 1 g/L A8-35 (□). In order to facilitate the comparison of the enthalpies in the presence and absence of BR, the *x*-axis refers to the concentration of free OTG in the samples injected. (B) Difference between the enthalpy of transfer of BR/OTG and that of pure OTG in (Δ) 1 g/L C22-43, (□) 1 g/L A8-35, (▲) 5 g/L C22-43, and (■) 5 g/L A8-35. Temperature: 25°C.

Remarkably, the enthalpy vs. [BR] traces are nearly identical in all cases, independently of the structure of the polymer and of its concentration in the cell (1 g/L or 5 g/L). The fact that the polymer concentration does not affect the enthalpy of association suggests that an excess of amphipol was present throughout the titration and that complete conversion to BR/polymer complexes was achieved. Invariance with polymer structure was not expected, though it points to some similarity in the origin (presumably hydrophobic) of BR trapping, irrespective of the exact structural details of the hydrophilic moieties in the polymer. The ITC measurements provide a strong indication of the similarity of the properties of A8-35 and C22-43 in terms of complexation energetics.

2.5.4. Stability of amphipol/BR complexes

Whenever sedimentation in sucrose gradients yielded a well-defined band of BR/polymer complexes, (C60-44, C60-30, C22-43, and A8-35), it was collected and its UV-visible spectrum was examined as a function of storage time. Judging from previous observations with IMP/anionic amphipol complexes (Tribet *et al.*, 1997), it is likely that detergent depletion was extensive. Thus the purified complexes are suitable objects for the assessment of the extent to which the polymer layer protects the protein from aggregation and inactivation. All BR/polymer complexes exhibited the absorption spectrum of native BR (Figure 2.4) and were devoid of free retinal, as indicated by the absence of an absorbance peak at 382 nm. Upon storage at 4°C in the dark, the spectrum of BR was invariant over a period of 13 days at pH 6.8.

At low pH or in the presence of high salt or calcium ions, A8-35 and IMP/A8-35 complexes tend to form aggregates (refs. (Gohon *et al.*, 2006; Picard *et al.*, 2006; Gohon *et al.*, 2007) and unpublished data). We tested the ability of PC-amphipols to preserve IMPs from aggregation under such conditions. Aliquots of purified BR/C22-43 and BR/A8-35 solutions (the two most concentrated fractions after sedimentation in sucrose gradients) were supplemented with either a NaCl solution, a citric acid-NaOH buffer, or a $CaCl_2$ solution to reach final concentrations of 1 M NaCl, 25 mM citrate, pH 5, and 12 mM Ca^{2+}, respectively (cf. *section 2.4*). Immediately after mixing (t_0), UV-visible spectra of the samples were measured to determine whether their transparency was altered. All samples containing A8-35 became turbid. In the case of C22-43, the samples treated with NaCl and those brought to low pH remained clear, while that supplemented with Ca^{2+} became turbid (Table 2.2). After 15 min. or 4 h of incubation at 20°C, the samples were centrifuged (10 min. at ~200,000 × *g*) and UV-visible spectra of the supernatants were recorded to determine the amount of protein present (Table 2.2). The PC-amphipols were highly efficient at maintaining more than 90% of the BR soluble in all cases. The turbidity observed in the presence of calcium was presumably due to the formation of calcium phosphate, judging from the presence of a white precipitate at the bottom of the centrifugation tubes. In contrast, a purple pellet formed upon centrifugation of the BR/A8-35 samples that had been treated with calcium ions or subjected to low pH conditions. A significant fraction of BR/A8-35 complexes, on the other hand, remained soluble in 1 M NaCl.

Table 2.2. Stability of IMP/amphipol complexes in presence of salts and at low pH.

Conditions	NaCl 1 M	pH 5	Ca^{2+} 12 mM	NaCl 1 M	pH 5	Ca^{2+} 12 mM	NaCl 1 M	pH 5
Amphipol	A8-35			C22-43			C60-44	
Turbidity at $t = 0$	+	+	+	-	-	+ [a]	-	-
% BR in supernatant $t = 15$ min	80	0	22	91	97	94	n.d.	n.d.
% BR in supernatant $t = 4$ h	79	0	6	93	94	92	93	94
% b_6f in supernatant $t = 1$ h							78	77

[a] A white precipitate forms (presumably calcium phosphate) upon addition of $CaCl_2$

Similar experiments carried out with b_6f/C60-44 complexes in the same buffers showed similar preservation of solubility in either 1 M NaCl or 25 mM citrate buffer (Table 2.2).

2.6. Discussion.

While tens, probably hundreds of detergents have been tested for their usefulness in membrane biochemistry (see e.g. refs. (le Maire *et al.*, 2000; Garavito & Ferguson-Miller, 2001), and references therein), only a handful of amphiphilic polymers have been hitherto applied to keeping IMPs soluble (Tribet *et al.*, 1996; Prata *et al.*, 2001; Sanders *et al.*, 2002; Picard *et al.*, 2006). Developing a range of alternative structures is of interest on many counts. For one thing, comparison of the structures of inefficient vs. efficient polymers will help delineating general rules for their design. Thus, most current studies suggest that the dispersity in size of the self-associated structures formed by A8-35 tends to correlate with that of IMP/A8-35 complexes (Picard *et al.*, 2006; Gohon *et al.*, 2007; Zoonens *et al.*, 2007). A similar observation is reported here for complexes of BR and PC-amphipols. This effect is seen –none too surprisingly– to depend on the size of the protein: monomers and dimers of cytochrome b_6f (~87 and ~215 kDa, respectively) were still relatively well resolved following complexation with the rather polydisperse C60-44 PC-amphipol, consistent with the view that the polymer represents a smaller fraction of the total bulk and mass of the particle than in the case of BR (~27 kDa) (cf. ref. (Tribet *et al.*, 1997)).

A number of similarities between IMP/A8-35 and IMP/PC-amphipol complexes, especially C22-43, were anticipated given that the two polymers share the same general structure. The fact that the energetics of BR transfer from OTG to amphipols appear nearly identical for A8-35 and C22-43 seemed surprising at first, however, given the very different nature of the hydrophilic groups of two polymers. This observation emphasizes the importance of hydrophobic interactions in the mechanism of complexation of IMPs by APols, consistent with the fact that these polymers seem to interact preferentially with the transmembrane surface of the protein (Zoonens *et al.*, 2005). ITC measurements provide new insights into the properties of self-association and complexation of amphipols, which may prove particularly relevant to understanding both IMP-associated amphipol layers and pure amphipol particles. Further ITC experiments are in progress and will be reported elsewhere.

Another incentive for the development of new amphipols is to provide the biochemist and biophysicist with a variety of molecules with complementary ranges of applications and conditions of uses. Examples of applications abound, where a diversification of amphipol structures and physical-chemical properties would be desirable. We may cite the following situations:

i) The best-characterized amphipol to date, A8-35, has repeatedly proven its efficiency at biochemically stabilizing the functional state of IMPs, e.g. BR or the sarcoplasmic calcium pump (Tribet *et al.*, 1996; Champeil *et al.*, 2000; Picard *et al.*, 2006). It cannot be used, however, in acidic media nor in the presence of calcium ions, if the monodispersity of the particles is to be preserved (Picard *et al.*, 2006; Zoonens *et al.*, 2007).

ii) IMP/A8-35 complexes can be studied by NMR (Zoonens *et al.*, 2005), but not at the slightly acidic pH that is optimal for most NMR studies involving amide protons.

iii) A8-35 provides an excellent medium for IMP renaturation (Pocanschi *et al.*, 2006) – an issue of great importance given the difficulty to overproduce IMPs in their native state – but its pH-sensitivity limits the range of refolding conditions that can be explored.

In all such cases, having access to pH-insensitive structures would broaden the range of applicability of amphipols. While organic chemistry suggests many possibilities, physical chemistry imposes rather severe constraints. Indeed, because a high density of grafting with hydrophobic chains is essential to the efficiency of amphipols, the polar moieties that keep the polymer soluble have to be highly hydrophilic. In addition to the original carboxylates (Tribet *et al.*, 1996), this had been achieved to date thanks to multiple hydroxyle groups (Prata *et al.*, 2001), a combination of quaternary amines and carboxylates (Gorzelle *et al.*, 2002), or sulfonates (Picard *et al.*, 2006; Dahmane *et al.*, 2007). While all of these amphipols have been shown to be able to maintain IMPs soluble and native, none of them have been thoroughly optimized nor characterized in great details yet.

2.7. Conclusion

The present work establishes the usefulness of a new family of amphipols, PC-amphipols, whose solubility is ensured by phosphorylcholine groups. As shown here, this type of polar group, which is highly soluble over a very wide range of conditions, including low pH or the presence of Ca^{2+} ions, makes it possible to prepare amphipols that have the potential to overcome many of the physical-chemical limitations of A8-35. In addition, the phosphorylcholine group is a good mimic of the polar moiety of phosphatidylcholine, which comprises a large proportion of membrane lipids in eukaryotic organisms. The PC moiety is likely to endow the complexes with particularly good biocompatibility.

2.8. Supporting information available (Appendix A)

Raw data corresponding to the enthalpograms shown in Figure 2.7. Also presented are enthalpograms corresponding to the titration of a buffer comprising 100 mM NaCl into *(a)* the same buffer and *(b)* a buffer with 95 mM NaCl. This material is available free of charge via the Internet at http://sciencedirect.com.

2.9. Acknowledgements

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CHAPTER THREE

RESEARCH PAPER

Enthalpy of Interaction and Binding Isotherms of Non-ionic Surfactants onto Micellar Amphiphilic Polymers (Amphipols).

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3.1. Abstract

The interactions in water between short amphiphilic macromomolecules, known as amphipols, and three neutral surfactants (detergents), dodecylmaltoside (DM), *n*-octylthioglucoside (OTG), and *n*-octyltetraethyleneoxide (C8E4) have been assessed by static and dynamic light-scattering (SLS and DLS), capillary electrophoresis (CE), and isothermal titration calorimetry (ITC). The amphipols selected are random copolymers of the hydrophobic *n*-octylacrylamide (25-30 mol %), a charged hydrophilic monomer, either acrylic acid (~35 mol %) or a phosphorylcholine-modified acrylamide (40 to 70 mol %) and, optionally, N-isopropylacrylamide (30 to 40 mol %). In water the copolymers form micelles of small size (hydrodynamic radius: ~5 nm). Neutral surfactants, below their critical micellar concentration (cmc), form mixed micelles with the amphipols irrespective of the chemical structure of the detergent or the polymer. The fraction of detergent in the surfactant/polymer complexes increases significantly (cooperatively) as the surfactant concentration nears the cmc. The ITC data, together with data gathered by CE, were fitted via the regular mixing model, which allowed us to predict the detergent concentration in equilibrium with complexes and the heat evolved upon transfer of detergent from water into a mixed surfactant/polymer complex. The enthalpy of transfer was found to be almost equal to the enthalpy of micellization, and the regular mixing model points to a near-ideal mixing behavior for all systems. Amphipols are promising tools in biochemistry where they are used, together with neutral surfactants, for the stabilization and handling of proteins. This study provides guidelines for the optimization of current protein purification protocols and

for the formulations of surfactant/polymer systems used in pharmaceutics, cosmetics, and foodstuff.

3.2. Keywords

Hydrophobically modified polymers; Surfactants; Amphipols; Isothermal titration calorimetry; Mixed micelles.

3.3. Introduction

The association of polymers with surfactants has captivated the interest of numerous scientists motivated by the possibility of coupling intrinsic solution properties of macromolecules, such as viscosity enhancement and stable coating of interfaces, with characteristic features of surfactants, such as their ability to self-assemble and to solubilize hydrophobes in water (Nagarajan, 1989; Goddard & Ananthapadmanabhan, 1992; Hansson & Lindman, 1996; Linse *et al.*, 1998; [a]Goddard & Gruber, 1999; La Mesa, 2005). From a practical standpoint, polymer/surfactants systems are incorporated in many industrial fluids, such as those used to enhance the oil recovery process, in paints, inks and coating fluids, as well as in food formulations, cosmetics ([b]Goddard & Gruber, 1999), and pharmaceutical preparations. To achieve tight binding and good synergy of properties, the mixed systems often consist of ionic surfactants bound via electrostatic interactions to oppositely charged polymers. Charged surfactants also bind to neutral polymers, although the association of cationic surfactants with non-ionic polymers tends to be weak (Hayakawa & Kwak, 1991). It is usually acknowledged that neutral surfactants either do not bind to polymers or that their interactions are so weak that they are difficult to detect by standard analytical tools (Brackman & Engberts, 1993). Polymers endowed of specific structural features display some affinity towards some neutral surfactants. For example, weakly dissociating polyacids, such as poly(acrylic acid), can associate to the polar head group of neutral detergents via hydrogen bonding (Saito & Angel, 1998; Anghel *et al.*, 1999; Robb & Stevenson, 2000; Ladhe *et al.*, 2006). Also, polymers carrying hydrophobic groups, such as polysoaps, hydrophobically modified polyampholytes (Sarrazincartalas *et al.*, 1994; Iliopoulos, 1998; [a]Miyazawa & Winnik, 2003; [b]Miyazawa & Winnik, 2003), polybetaines (Johnson *et al.*, 2004) or modified cellulosics (Winnik, 1990), can interact with neutral surfactants, as they offer additional attraction between polymer-linked hydrophobes and surfactant tails (Winnik *et al.*, 1991; Balazs *et al.*, 1993; Barreiro-Iglesias *et al.*, 2003). Neutral surfactants are commonly used in biology as well as in cosmetics and pharmaceutical formulations in view of their mildness and low toxicity. Neutral surfactants are essential in protocols of protein extraction and purification and, specifically, for the isolation of integral membrane proteins (IMP), a major class of proteins that are targeted by the majority of current drugs (Le Maire *et al.*, 2000; Sanders & Oxenoid, 2000). A combination of neutral surfactants (called detergents) and specific amphiphilic polymers, known as amphipols (Popot *et al.*, 2003; Sanders *et al.*, 2004) or peptitergents (McGregor *et al.*, 2003), was shown to facilitate the handling of IMPs and to improve IMPs' stability. From an experimental standpoint, the purification and isolation of IMPs with amphipols require one to adjust with care, and mostly empirically, the concentrations of detergents, lipids, and polymers that are used to extract the protein from the membrane and to stabilize the protein in solution. The protein to be purified is entrapped in detergent micelles prior to formation of a mixed protein/polymer/detergent assembly. Then, the detergent and the excess polymer are removed and the protein is recovered as a stable amphipol complex (Popot *et al.*, 2003; Sanders *et al.*, 2004). At each step of the purification process, exchange of surfactant between protein and amphipol takes place, leading to the formation of amphipol/detergent complexes, which eventually are separated from the desired IMP/amphipol complexes (Tribet *et al.*, 1997). While IMP/amphipol complexes have been characterized thoroughly (Zoonens *et al.*, 2005), little attention has been paid so far to the amphipol/detergent complexes formed in the intermediate stages of the purification process, although it has been suggested that the formation of amphipol/detergent complexes may affect the stability and the enzyme activity of IMPs (Champeil *et al.*, 2000). To devise robust protocols, one needs to establish surfactant/amphipol binding isotherms, to determine the composition of mixed micelles, and to elaborate models allowing one to predict the composition of mixed micelles with varying amphiphile concentrations. Such data are currently unavailable since in most previous studies dedicated to neutral surfactant/polymer interactions, conclusions about binding strength were based primarily, or solely, on the magnitude of the polymer-induced decrease of the surfactant critical micelle concentration (cmc), assessed by NMR and EPR spectroscopy, small-angle scattering (neutron, X-ray, light), microcalorimetry, and fluorescence spectroscopy

(Drummond *et al.*, 1992; Aoudia & Zana, 1998; Hashidzume *et al.*, 1999; Couderc *et al.*, 2001) or on viscosity enhancement (Sarrazincartalas *et al.*, 1994; Johnson *et al.*, 2004).

We report here an analysis of neutral surfactant/amphipol systems with three complementary techniques: dynamic and static light-scattering (DLS and SLS), isothermal titration calorimetry (ITC), and capillary electrophoresis (CE). Light scattering measurements were carried out to assess the occurrence of detergent-induced changes in the micellar size of aqueous amphipols. ITC gave access to the thermodynamic parameters associated with the binding process. It provided a highly sensitive signature of the association, which was analyzed using the binding isotherms obtained by CE. The systems under scrutiny comprise three neutral detergents of common use in biology, *n*-octylthioglucoside (OTG), *n*-octyltetraethyleneoxide (C8E4), and *n*-dodecylmaltoside (DM), and three amphipols (Figure 3.1 and Table 3.1), the hydrophobically modified poly(acrylic acid) A8-35, currently employed as a tool in biochemistry, and two phosphorylcholine (PC)-based amphiphilic polymers, C22-43 and C45-68, which are prototypes of a new class of amphipols developed recently in our groups (According to the amphipol nomenclature used in refs (Tribet *et al.*, 1997; Popot *et al.*, 2003), A and C stand for anionic and cationic, the digit adjacent to the letter refers to the average molar mass (in kg mol^{-1}), and the second digit is the molar percent of carboxylate or phosphorylcholine residues, respectively. Note that the PC-modified polymers are cationic under the conditions of our measurements as a consequence of the presence

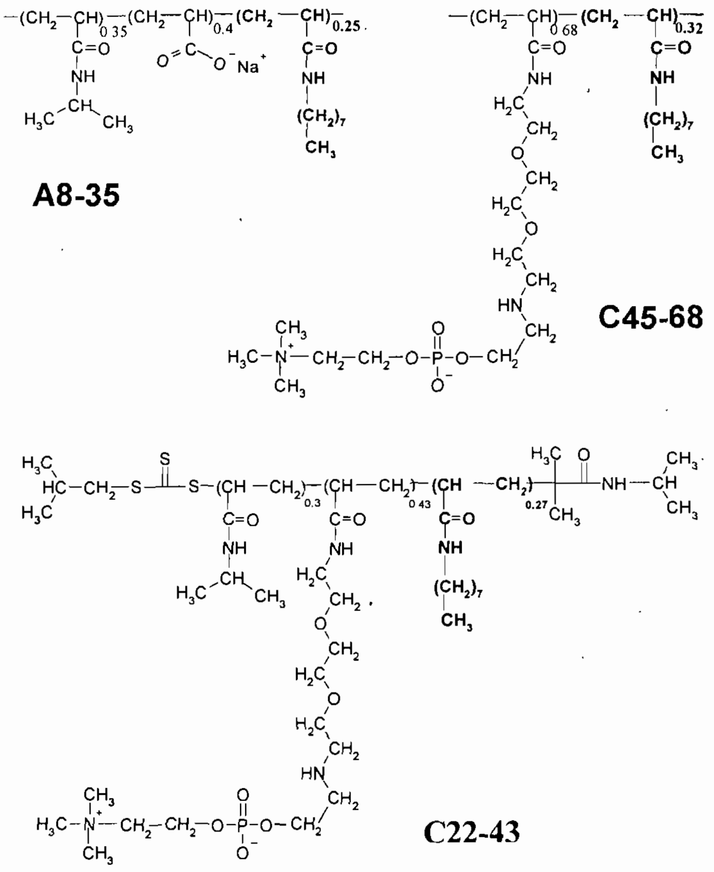

Figure 3.1: Chemical structure of the amphipols investigated.

Table 3.1. Characteristics of the polymers employed in the study.

	Composition (mol %)					
Polymer	NIPAM	AA[a]	PC	C_8H_{17}	M_n (g/mol)	M_w/M_n
A8-35	40[b]	35[b]		25[b]	7500	1.6
C22-43	30[c]	--	43[c]	27[c]	22000[c]	1.2
C45-68	--	--	68[c]	32[c]	45000[c]	2.5

[a] AA: acrylic acid; NIPAM: *N*-isopropylacrylamide; PC: *N*-phosphorylcholine-*N'*-[(ethylenedioxybis(ethyl) acrylamide]; and C_8H_{17}: *n*-octylacrylamide.

[b] See refs (Tribet *et al.*, 1997; Gohon *et al.*, 2006).

[c] See Supporting Information (Appendix B).

of a secondary amine in the fragment linking the PC group to the polymer main chain). The amphipols A8-35 and C22-43 are random terpolymers of *n*-octylacrylamide, *N*-isopropylacrylamide, and either acrylic acid (A8-35) or *N*-phosphorylcholine-*N'*-ethylenedioxybis(ethyl)acrylamide (C22-43), while the amphipol C45-68 is a random copolymer of *n*-octylacrylamide and *N*-phosphorylcholine-*N'*-ethylenedioxybis(ethyl)acrylamide. To unify data collected from different systems, we used the regular mixing model to analyze the binding energy and the binding isotherms between free detergent and polymer-bound detergent. This approach allowed us to assess the contribution of chemical parameters on the association mechanism. Our results will enable the development of improved methodologies in structural biology and, more importantly, they will enhance the current understanding of amphiphilic polymer/neutral detergent systems.

3.4. Experimental Procedures

3.4.1. Materials

Water was deionized with a Millipore Milli-Q water purification system. *n*-Octyltetraethyleneoxide (99%) (C8E4) was purchased from Bachem. *n*-Dodecyl maltoside (98 %, DM), and *n*-octylthioglucoside (98 %, OTG) were purchased from Sigma Chemicals and used without further purification. The amphipol A8-35 was prepared as reported earlier (Gohon *et al.*, 2006). The polymers C22-43 and C45-68 were prepared by slight modifications of reported procedures. Details of their synthesis and characterization are given as supporting information (Appendix B).

3.4.2. Instrumentation

3.4.2.1. Light-scattering

Static (SLS) and dynamic (DLS) light-scattering experiments were performed on a CGS-3 goniometer (ALV GmbH) equipped with a ALV/LSE-5003 multiple-τ digital correlator (ALV GmbH), a He-Ne laser (λ = 632 nm), and a C25P circulating water bath (Thermo Haake). The temperature was set at 25 °C. For DLS analysis, the homodyne intensity-intensity correlation function $G(q,t)$ was related to the correlation function of the scattered field by equation 1

$$G(q,t) = A.(1 + b\, g^2(q,t)) \qquad \textbf{(1)}$$

where A is the experimental baseline and b is the fraction of correlated light. Inversion of the autocorrelation function g was performed using the program CONTIN, providing a distribution of apparent Stokes radii.

3.4.2.2. Isothermal Titration Calorimetry (ITC)

Measurements were carried out with a VP-ITC instrument from Microcal Inc. Prior to each experiment, the sample cell (volume = 1.43 mL) was filled with water, buffer, or a polymer solution. The heat exchanges generated by surfactant/polymer interactions were obtained from titrations of surfactants aqueous solutions into aqueous polymer solutions (0-5.0 g L^{-1}). The surfactant solutions were placed in a 300 μL syringe continuously stirred (300 rpm) during the titration, allowing rapid mixing of the titrant in the sample cell (Blandamer *et al.*, 1998). Aliquots (2-10 μL) were injected into the sample cell in intervals of 300 s. All measurements were carried out at 25 °C. Each titration was performed twice to ensure reproducibility of the results. Data were analyzed using the Microcal ORIGIN software.

Data Treatment. The experimental enthalpy change h_i resulting from the *ith* surfactant solution injection was obtained by integration of the raw data signal. The integrated molar enthalpy change per injection (ΔH_i, in cal/mol of surfactant) was obtained by dividing h_i by the number of moles of surfactant added n_i; hence $\Delta H_i = {}^{h_i}\!/_{n_i}$, resulting in enthalpograms, which are plots of ΔH_i as a function of total surfactant concentration in the calorimeter sample cell.

3.4.2.3. Capillary electrophoresis

Experiments were carried out with a Beckman P/ACE system MDQ instrument equipped with a diode array multiwavelength UV-vis detector (Beckman Instruments) operating at 25°C in the 4-12 kV range and fitted with bare silica capillaries (75 μm × 31 cm, effective length : 21 cm; J and W Scientific). The capillary was flushed daily with 0.1 M NaOH, followed by a water rinse, and finally was allowed to equilibrate with the running buffer (100 mM NaCl, 20 mM NaH_2PO_4-NaOH, pH 7.0). For measurements with the cationic polymers C22-43 and C45-68, adsorption of the polymers on the capillary wall was prevented by treating the capillary with an aqueous 0.5% polyethylene oxide (PEO 20 000, Fluka) solution prior to equilibration with the buffer. Sample solutions were prepared by mixing an OTG stock solution (200 mM; 61.7 g/L) and a polymer stock solution (10 g/L) in the running buffer. The OTG concentration ranged from 0 to 30 mM while the polymer concentration was maintained constant (2.5 or 5.0 g/L). The solutions were kept at room temperature for at least 2 h prior to measurement. Frontal analysis was carried out using continuous electrokinetic injection mode against the running buffer, with a positive voltage (A8-35) or negative voltage (C22-43 and C45-68).

3.4.3. Solution preparation

Stock polymer solutions (2-20 g/L) were prepared by dissolving freeze-dried samples in deionised water under gentle stirring for at least 2 h at room temperature. The polymer solution in water was mixed with an adjusted volume of concentrated buffer (NaH_2PO_4-NaOH, pH 7.0) and NaCl to achieve a final concentration of 20 and 100 mM, respectively. The solutions were filtered through a 0.22 μm syringe filter (Millex, Millipore) prior to measurements. In some cases, samples were subjected to ultracentrifugation (200 000 g, 10min., Beckman Optima Max, MLA 130 rotor) prior to analysis to remove residual trace amounts of aggregates or dust particles.

3.5. Results

3.5.1. Light-scattering measurements

As a consequence of their high degree of hydrophobic modification, amphipols undergo self-association in water, a process that has been characterized in detail in the case of aqueous A8-35 in conditions similar to those used in the present study (Gohon *et al.*, 2006). Thus, in an aqueous NaCl/phosphate buffer of pH 7.0, the solvent employed throughout this study, the polymer A8-35 forms compact, micelle-like assemblies with a molar mass of ~ 40 kg mol^{-1} and a Stokes radius below ~5 nm (Gohon *et al.*, 2006). The size of these assemblies is independent of polymer concentration, at least within the experimentally accessible range from 1.0 to 20.0 g/L. Assemblies formed in aqueous A8-35, are paucimolecular, as they consist, in average, of no more than four macromolecules (i.e. 75-80 n-octyl chains). The PC-based amphipol C22-43 forms species having a Stokes radius of 4.4 ± 1.0 nm (Figure 3.2 a). This value is similar to the radius of detergent micelles in the same conditions (~3.3 nm for C8E4 micelles, and 4.0-5.5 nm for OTG slightly above its cmc (Molina-Bolivar *et al.*, 2006); see supporting information or appendix B). The amphipol C45-68 self-assembles in the buffer into larger objects, which have a Stokes radius of ~24 nm and a broad size distribution. The difference in the solution properties of the two PC-based amphipols is believed to derive from two structural factors: (i) C45-68, which was synthesized by standard free radical polymerization, has a larger molar mass and its molecular weight distribution is broader, as compared to C22-43, which was obtained by controlled free radical polymerization (Table 3.1) and (ii) unlike A8-35 and C22-43, the polymer C45-68 does not contain N-isopropylacrylamide units along its backbone. The impact of these structural differences on the properties of amphipols merits further investigation to delineate amphipol design rules. However, per se, the amphipol C45-68 is not suitable for use in biology, where amphipols of sharp micellar size distributions are required.

Next, we carried out SLS and DLS measurements with mixed amphipol/surfactant solutions, to assess the surfactant-induced changes in the molar mass and size of the amphipol assemblies. The absence of a significant contribution of interpolymer interaction to DLS and SLS, and the relevance of size distribution obtained at a scattering angle of 90°, has been validated in a previous investigation of the amphipol A8-35 (Gohon *et al.*, 2006). Solutions of constant polymer concentration, 2.5 or 5.0 g/L, were treated with increasing amounts of surfactants over a concentration range that includes their cmc. DLS measurements revealed that, in the presence of C8E4, the hydrodynamic radius of the polymer assemblies increased slightly upon addition of detergent (Figure 3.2a). The largest effect was observed in the case of the C8E4/A8-35 system for which the micellar Stokes radius increased by ~ 2.5 nm. Mixed micelles remained well defined and similar to virgin polymer assemblies (Figure 3.2a). Monomodal distributions were obtained over the entire surfactant concentration window probed (0-30 mM) in the case of mixed C8E4/C22-43, and C8E4/A8-35, and up to 9-12mM OTG in the presence of A8-35. Solutions of A8-35/OTG became turbid when [OTG] > 12 mM, and the polymer/surfactant assemblies increased in size to an extent such that light-scattering characterization became unreliable (Figure 3.2b). No phase separation was observed in the other mixed polymer/surfactant systems. It is important to note that virgin OTG micelles themselves are prone to elongation and growth with increasing concentration above the cmc (see Figure 3.1S, sup. Info. or appendix B).

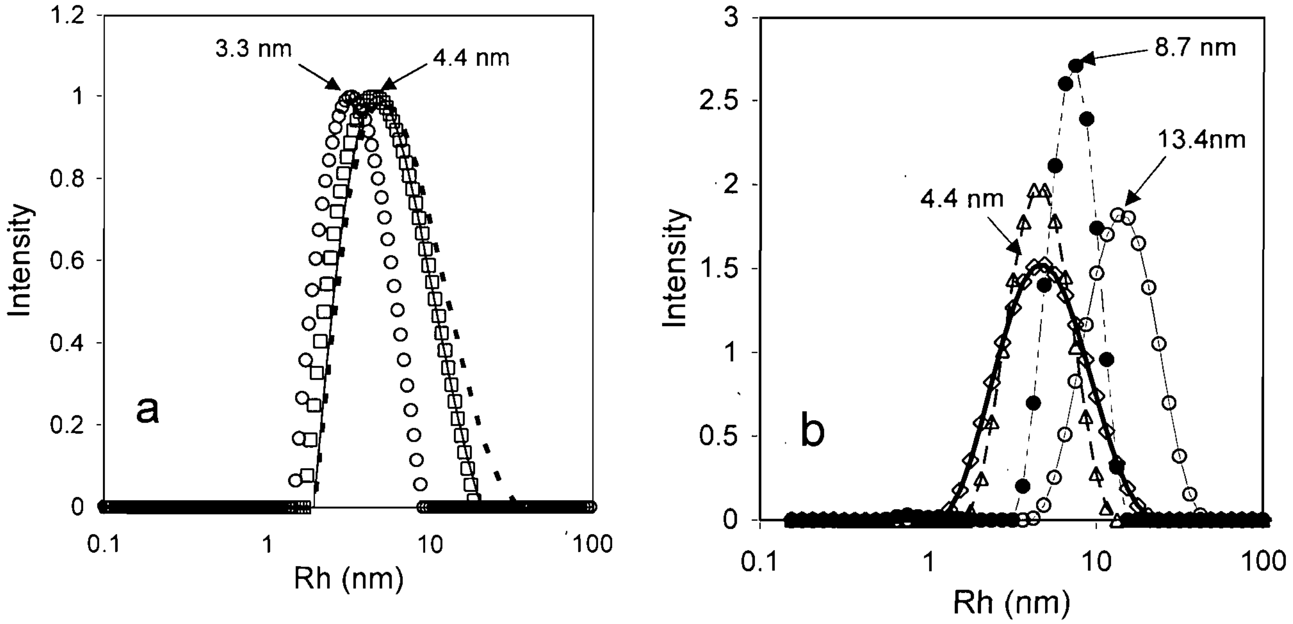

Figure 3.2 (a and b): (a) Hydrodynamic radii distribution obtained from DLS measurements carried out with solutions of 2.5 g/L C22-43 (○), or 17 mM C8E4 and no polymer (□), or mixtures of 2.5 g/L C22-43 and 5 mM (solid line) or 15mM total C8E4 (dashed line). (b) Hydrodynamic radii distribution obtained from DLS measurements carried out with solutions of A8-35 (◇) and mixtures of A8-35 with OTG of total concentration 6 mM (△), 12 mM (●) and 16mM (○); polymer concentration: 2.5 g/L.

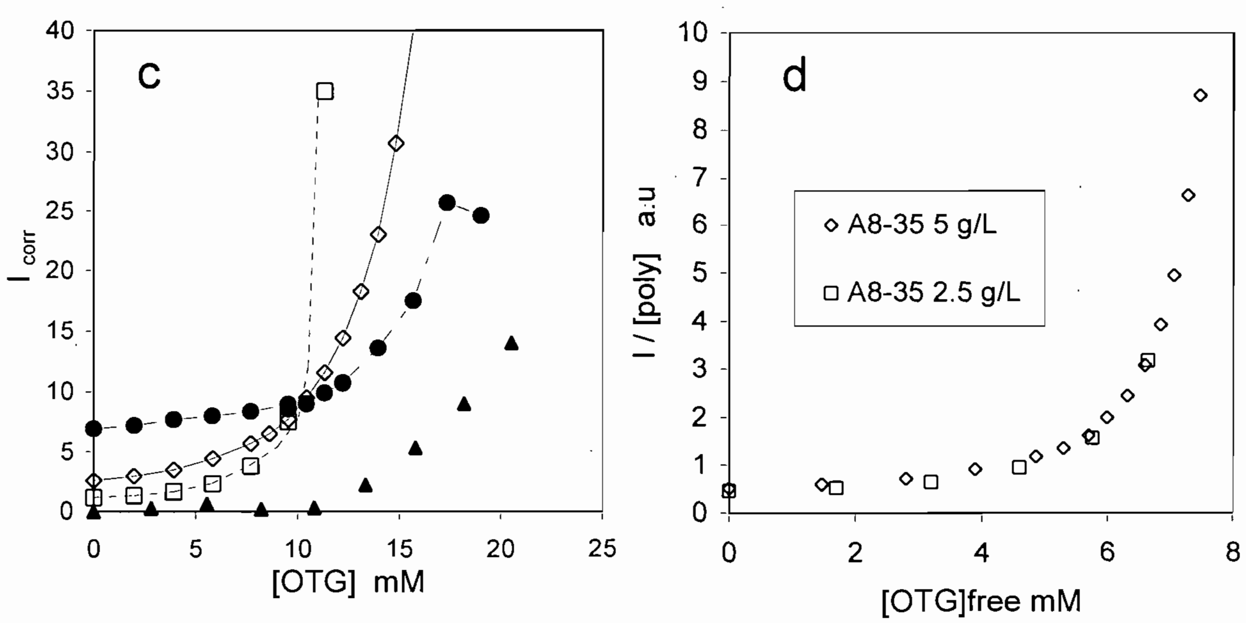

Figure 3.2 (c and d): (c) Plot of the scattering intensity measured by SLS as a function of total concentration of OTG for solutions of the surfactant alone (▲) and of surfactant in the presence of A8-35 (2.5 g/L; □), A8-35 (5.0 g/L; ◇) and C22-43 (2.5 g/L, ●). (d) Plot of the changes of the normalized scattered light intensity as a function of free OTG. Normalization of I (I_{corr}): data were corrected to account for the scattering of the solvent and normalized by the scattering of toluene. All measurements were carried out with solutions in a phosphate buffer [NaH_2PO_4-NaOH (20 mM) and NaCl (100 mM)]; pH: 7.0; temperature: 25 °C.

The intensity of light scattered by aqueous solutions of increasing detergent concentration was monitored by SLS for solutions of OTG and mixed solutions of OTG with either A8-35 or C22-43 (Figure 3.2c). For solutions of OTG in the absence of amphipol, the scattered light intensity remained constant until [OTG] reached ~10 mM. It increased sharply as the surfactant concentration exceeded this threshold, which is slightly higher than the CMC of OTG obtained by other techniques (e.g. 7.5 ± 0.5 mM in NaCl 100 mM) (Saito & Tsuchiya, 1984; Molina-Bolivar *et al.*, 2006). In contrast, for mixed amphipol/OTG solutions, the scattered light intensity increased slowly and regularly with increasing OTG concentrations much lower than the cmc. This significant enhancement of scattering intensity is indicative of the formation of amphipol/detergent complexes prior to detergent micellization. The OTG concentration for which the scattering intensity begins to increase sharply depends on the polymer structure and on its concentration. It is lower than 10 mM in the case of A8-35, which is also indicative of the formation of relatively large mixed assemblies below the cmc. The sharp increase in scattering intensity is observed at concentration slightly higher than the cmc in the case of C22-43. The phenomena contributing to scattering from mixed amphipol/OTG solutions include detergent binding, detergent micellization, and any variation of the average number of polymer chains involved either in self-assemblies or in mixed micelles. For mixed C22-43/OTG solutions, it is, in practice, difficult to determine the exact origin of the scattering above the cmc since the scattering intensity due to C22-43 itself is of the same order of magntitude as typical scattering by detergent micelles. Nevertheless, the linear increase of the scattering intensity below the cmc is a strong indication that C22-43/OTG complexes form below the cmc of OTG. In addition, the scattering intensity increases more rapidly in mixed systems (OTG/A8-35 samples), as compared to solutions of OTG alone. Similar trends in scattered light intensity were observed in mixed C8E4/A8-35 solutions for detergent concentrations below the cmc and above.

From the light-scattering experiments, we conclude that small amphipol/surfactant assemblies form in all mixed polymer/surfactant solutions. No macroscopic aggregation took place, in the mixed systems, except in the case of the OTG/A8-35 pair. Although the radius of the mixed assemblies increases with surfactant concentration, it does not differ markedly from that of isolated C8E4 or OTG micelles. The slow increase in scattered light intensity at low surfactant concentrations points to the formation of mixed assemblies, whose molar mass increases regularly, although weakly, well below the cmc of the surfactant. The sharper increase obtained when the (total) surfactant concentration approaches the cmc points to the cooperative nature of the binding. Interestingly, the intensity of the light scattered by mixtures of A8-35 and OTG can be rescaled to account for the density of scatterers (plot of intensity/[polymer]) and for the equilibrium with free (unbound) OTG (obtained from CE measurements). The intensity/[polymer] essentially depends on the concentration of free OTG (Figure 3.2d) with a rapid increase close to the cmc of OTG. The composition (and molar mass) of the complexes is controlled by the equilibrium with unbound surfactants in solution and to a much lesser extent by the polymer concentration.

3.5.2. Isothermal titration calorimetry

Aliquots of a micellar surfactant solution in a phosphate buffer of pH 7.0 were titrated at constant temperature (25 °C) into the same buffer or into an amphipol solution in this buffer. After each injection, heat was released as a result of the various processes occurring in solution. The enthalpy per injection was determined as a function of total surfactant concentration. Turning our attention first to the enthalpograms recorded in the absence of polymer, upon dilution of micellar solutions of OTG (100 mM), C8E4 (100 mM), and DM (2.5mM) in buffer (Figures 3.3 and 3.4 open circles), we note that the dilution of the surfactants is exothermic, until the surfactant concentration in the cell reaches 10 mM, 8.0 mM, and 0.2 mM for OTG, C8E4, and DM, respectively. When the surfactant concentration exceeds this value, the heat evolved decreases sharply, nearing a value close to zero (within a 1-2 mM concentration window for OTG and C8E4, or a 0.1mM window for DM). The concentration corresponding to the sharp transition between high exothermicity and weak effect is just above the cmc of each surfactant, measured by other techniques (7-8, 7-8.5, and 0.18 mM for solutions of OTG, C8E4, and DM, respectively) (Le Maire *et al.*, 2000; Molina-Bolivar *et al.*, 2006). The heat evolved in the system before reaching the cmc is mostly due to the demicellization of the surfactants, with some contribution of the heat of dilution of individual surfactant molecules and of the micelles. Contributions of the heat of micelle dilution can be neglected in view of the near 0 heat of dilution recorded in the micellar sections of the enthalpograms.

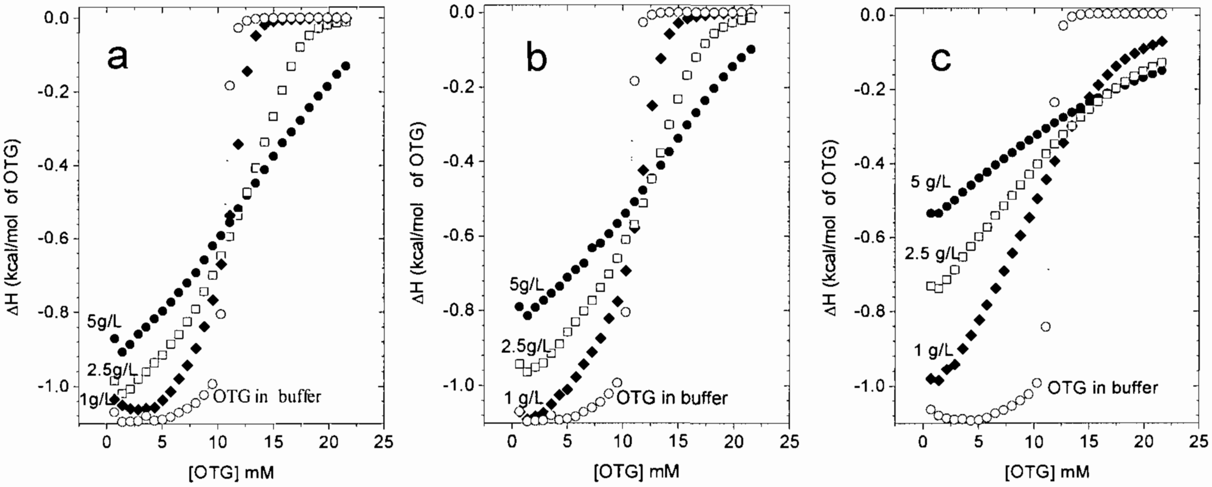

Figure 3.3: Plots of the changes of the enthalpy of dilution (Δ*H*) of OTG (100 mM) into buffer (O) and of the enthalpy of transfer of OTG (100 mM) into (a) solutions of C45-68, (b) solutions of C22-43, and (c) solutions of A8-35. Polymer concentration: 1.0 g/L (◆), 2.5 g/L (□), and 5.0 g/L (●) as a function of total OTG concentration; solvent: phosphate buffer [NaH_2PO_4-NaOH (20 mM) and NaCl (100 mM)]; pH: 7.0; temperature: 25 °C.

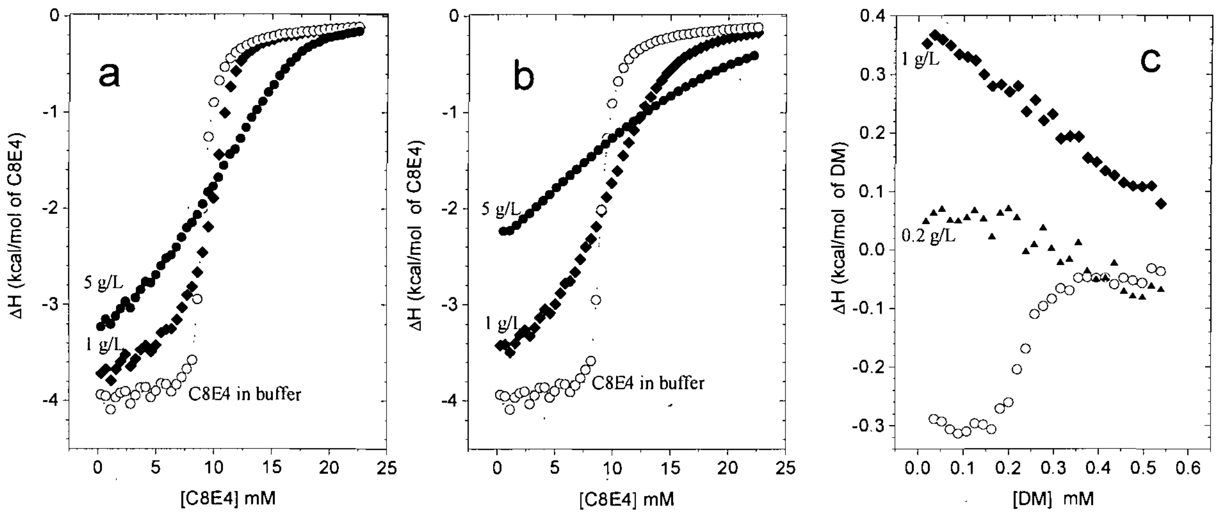

Figure 3.4: Plots of the changes of the enthalpy of dilution (Δ*H*) of C8E4 (100 mM) into buffer (○, a and b) and of DM (2.5 mM) into buffer (○, c) and of the enthalpy of transfer of C8E4 (100 mM) into (a) solutions of C22-43, and (b) solutions of A8-35, and (c) of the enthalpy of transfer of DM (2.5 mM) into solutions of C22-43. Polymer concentration: 1.0 g/L (◆) and 5.0 g/L (●) as a function of the total detergent concentration; solvent: phosphate buffer [NaH_2PO_4-NaOH (20 mM) and NaCl (100 mM)]; pH: 7.0; temperature: 25 ^{o}C.

Since approximately 90-92 mol% of either OTG or C8E4 is in the form of micelles in the syringe (100 mM solutions), the heat of dilution measured by ITC for surfactant concentrations lower than the cmc (approximately -1100 cal/mol and approximately -4000 cal/mol for OTG and C8E4, respectively) corresponds to ~ 90% of the heat of demicellization of each surfactant. Note that the demicellization enthalpy of OTG, approximately -1300 cal/mol (-5400 J/mol), is significantly lower than that of C8E4 (approximately -4700 cal/mol = -19600 J/mol from figure 3.4a) but substantially higher than that of DM (approximately -330 cal/mol = -1400 J/mol) (Figure 3.4c, open circles).

In the presence of polymers, the heat evolved upon injection of a micellar surfactant solution reflects not only the heat of demicellization, but also the enthalpy of interaction between the surfactant and the polymer. The heat evolved upon surfactant injection into polymer solutions was significantly less than in the absence of polymer, even for the lowest surfactant concentration domains probed (cmc/30 < c < cmc/10) pointing to an endothermic contribution attributable to the energy of binding of surfactant onto polymer (Figures 3.3 and 3.4). The magnitude of the effect increases with increasing polymer concentration, implying that surfactant binding occurs in proportion to the amount of polymer chains in solution. The endothermic contribution is the highest in the case of the amphipol A8-35, as compared to C45-68 and C22-43 of identical concentration, irrespective of the detergent used. It is important to remember that due to large differences in the molar mass of the repeat units of the three polymers, similar polymer concentrations by weight can differ by a factor of ~2 in terms of mol % concentration of octyl side groups. Since the repeat units of C45-68 and C22-43 are of similar mass, a qualitative comparison is justified in this case, and it is pertinent to note that binding of surfactant to the more hydrophobic polymer C22-43 produces a more endothermic effect, hinting to the prevalent contribution of hydrophobic interactions between surfactant tails and polymer-linked hydrophobes. Nonetheless, a quantitative analysis of binding isotherms in terms of molar binding degree is required to discuss the binding strength in terms of polymer structure (see Discussion).

In the section of the enthalpograms corresponding to a total surfactant concentration higher than the cmc, the heat evolved upon injection of surfactant into aqueous polymer solutions is different from that recorded upon dilution of detergent into buffer. In this range of concentrations, differential enthalpy curves (curve in the presence of polymer minus curve in its absence) are often used to characterize critical binding parameters, such as the critical insertion concentration (cic) and the critical aggregation concentration (cac) (Seng *et al.*, 2000; Tam *et al.*, 2000). In the cases discussed here, however, using the enthalpogram of surfactant above the cmc as a blank curve is questionable. Recall that we observed that amphipol/detergent assemblies are present over the entire experimental concentration window and that amphipol can self-assemble in micelle-like clusters. Since hydrophobic clusters are present irrespective of surfactant concentration, a fraction of the surfactant molecules is likely to transfer from water into the micelle-like clusters, irrespective of the magnitude of the surfactant concentration and its closeness to the cmc. Assuming that a significant fraction of the surfactant is bound to the polymer at all times, the concentration of free surfactant will be lower than the total concentration represented in the *x*-axis throughout the enthalpogram. Full demicellization occurs accordingly in samples for which the total concentration of detergent is above the cmc, such that the correct blank to be subtracted from the mixed system enthalpogram over the entire concentration range is the enthalpy of demicellization and possible minor terms accounting for dilution of micelles and detergent monomer. This analysis is consistent with the absence of discontinuity in the 8-12 mM region of the curves corresponding to dilutions in polymer solutions of concentrations 2.5-5 g/L (Figures 3.3 and 3.4). The situation may be somewhat different in the case of dilutions into less concentrated C45-68 and C22-43 solutions (1 g/L, Figures 3.3 and 3.4, ◆), for which the abrupt upward decrease of the heat evolved near the cmc is only weakly dampened, as compared to surfactant demicellization. In all other cases, the continuity of the curves as a function of detergent concentration indicates that abrupt co-micellization does not occur.

3.5.3. Capillary electrophoresis

The binding of detergent onto amphipols was investigated by capillary electrophoresis under the same conditions as those selected in the ITC investigation using a CE protocol employed previously to analyze similar systems (Collet *et al.*, 1996; Gao *et al.*, 1997). The method allows one to measure the concentration of free (unbound) surfactant in equilibrium with amphipol-bound surfactant. In principle, it can be applied to any detergent detectable by the CE system. In our case, we used a UV-vis detector, and of the three surfactants used, only OTG possesses sufficient intrinsic absorbance for detection. Consequently, we limited the CE studies to amphipols/OTG systems. Neither free OTG nor OTG micelles are charged; however OTG/amphipol complexes are charged given the ionic nature of the polymers in the pH conditions of the measurements. Consequently, the electrophoretic mobilities of OTG/amphipols complexes and of unbound OTG or its micelles will be markedly different. Under conditions of continuous sample injection in the CE capillary, a sharp zone of unbound OTG, dragged by the injection flow, forms in advance of the zone of polymer/surfactant complexes, the motion of which is retarded by the counter-flow. The first increase in absorbance in the elution profile of the mixed OTG/A8-35 system (Figure 3.5) corresponds to the outflow of unbound OTG, at a mobility of $0 \pm 1\ 10^{-5}$ cm^2/V s. The subsequent short plateau is due to the elution of the zone containing unbound OTG. The height of this plateau is proportional to the concentration of unbound OTG. Using this value, taken as the inflection point of the electropherograms (arrow in Figure 3.5), together with a calibration curve drawn using OTG standard solutions, one can determine $[OTG]_{free}$. The elution profile presents a second increase in absorbance for a mobility that varies depending on the composition of the OTG/polymer sample. For example, increasing the total concentration of OTG in mixed OTG/A8-35 samples from 3 mM to 16 mM, keeping the polymer concentration constant, resulted in a decrease of ~5 x 10^{-5} cm^2/Vs in the electrophoretic mobility of the complexes (Figure 3.5). The electrophoretic mobility of the complex changes as a function of $[OTG]_{free}$ are presented in Figure 3.6a for two OTG/amphipol systems.

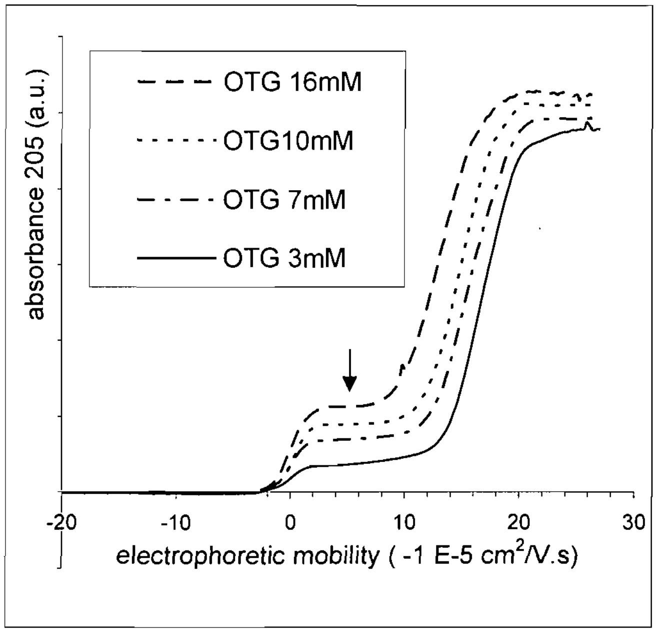

Figure 3.5: FACCE electropherograms recorded for mixed OTG/A8-35 solutions of constant polymer concentration (2.5 g/L) and various total OTG concentrations; solvent: phosphate buffer [NaH_2PO_4-NaOH (20 mM) and NaCl (100 mM)]; pH 7.0; temperature: 25 °C. The arrow points to the electrophoretic mobility value for which the $[OTG]_{free}$ value was determined experimentally (the corresponding $[OTG]_{free}$ values were: 2.83, 5.43, 6.98, and 8.80 mM).

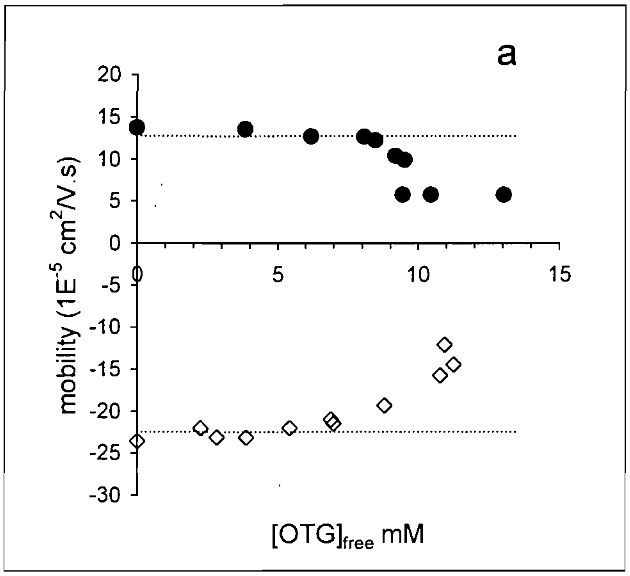

Figure 3.6: **(a)** Plots of the changes in electrophoretic mobility of polymer/OTG complexes as a function of the concentration of free OTG (◇): A8-35; (●): C22-43; polymer concentration: 2.5 g/L.

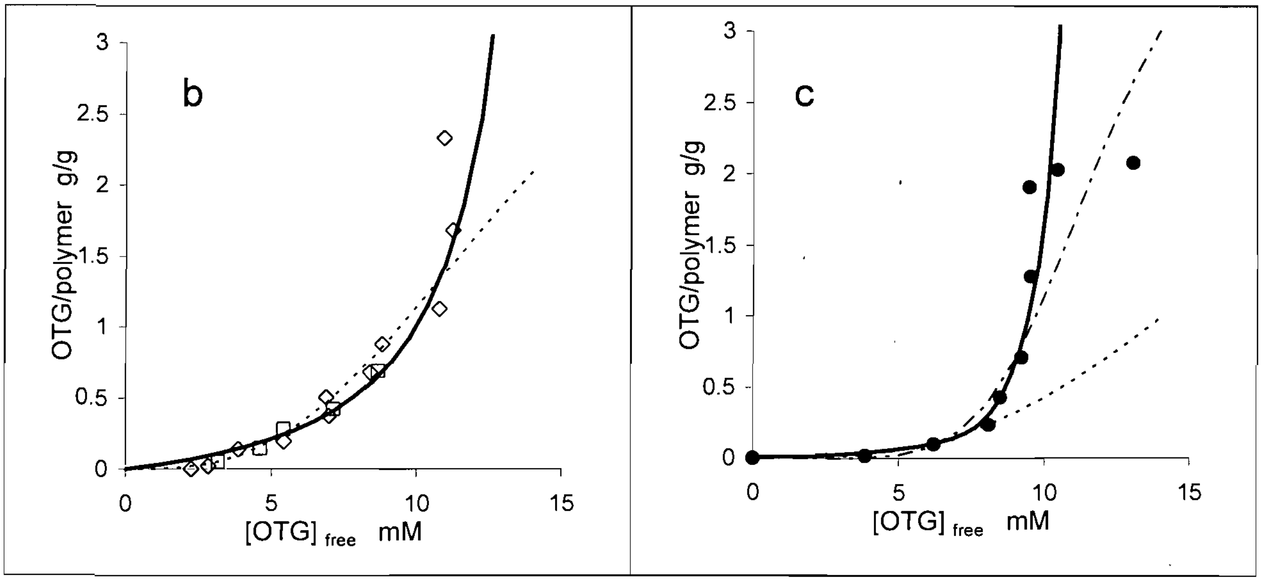

Figure 3.6: (b and c) Plots of the changes of the mean weight ratio of OTG bound to the polymer as a function of the concentration of free OTG. The dashed lines reflect the calculated fits of the data using the Hill model (eq 2, n = 4), whereas the full line reflects the calculated fits of the data using the regular solution model (eq 3). (b) A8-35 (◇): 2.5 g/L; (□): 5 g/L; and parameters ($K = 4.10^{-4}$, α =3, A= 15 mM, β= 0.5) ; (c) C22-43 (●): 2.5 g/L and parameters ($K = 1.2.10^{-4}$, α = 3) dashed line or ($K = 4 \times 10^{-7}$, α = 6) dash-dotted line, and (A = 11.5 mM, β= 1.4) solid line.

Micelles of A8-35, in the absence of OTG, bear a negative charge, as expected for a copolymer of acrylic acid in a pH 7 buffer, whereas micelles of C22-43 bear a positive charge, reflecting the partial protonation of the secondary amine group that links the PC substituent to the polymer main chain. The electorophoretic mobility of each polymer remains constant in the presence of low concentrations of OTG, but it shifts toward neutral values as $[OTG]_{free}$ nears the cmc of OTG. This decrease in electrophoretic mobility reflects the enhanced friction experienced by the polymer as it drags more and more quasi-neutral micelles (Collet *et al.*, 1996; Gao *et al.*, 1997).

Binding isotherms corresponding to the adsorption of OTG to A8-35 and C22-43 (polymer concentration: 2.5 and 5.0 g/L) are presented in Figures 3.6b, c, respectively, where the $[OTG]_{free}$ values were determined experimentally from the CE elution profiles. In all cases, the amount of polymer-bound OTG increases sharply as $[OTG]_{free}$ nears the cmc of OTG. It reaches values exceeding 2g/g OTG/polymer over a narrow $[OTG]_{free}$ window. The isotherms in Figure 3.6b do not depend on polymer concentration, suggesting that the interactions follow a binding model for which the detergent binding sites do not change significantly upon polymer dilution. The data rule out a model involving the formation of mixed micelles with varying numbers of polymer chains since in such a case, the data should exhibit a significant polymer concentration dependence.

The rapid increase of binding with increasing OTG concentration beyond a threshold value may be taken as an indication that there exists a critical [OTG] for association to take place, the critical interaction concentration (cic) often invoked in studies of polymer/surfactants systems in which polymers are not micellized (Konop & Colby, 1999; Li *et al.*, 1999; Colby *et al.*, 2001). However, our data are also compatible with a model of continuous, although cooperative, variations of binding. To test the cooperative nature of the OTG/amphipol binding mechanism, we set about to assess the quality of the fit of the experimental data with two models, the Hill isotherm model and the regular mixing model, which are appropriate models for multisite binding with cooperativity. The Hill isotherm model provides the following expression:

$$\frac{[OTG]_{bound}}{[poly]_{tot}} = nK\frac{[OTG]^{\alpha}_{free}}{1+K[OTG]^{\alpha}_{free}} \qquad \textbf{(2)}$$

where $[\mathrm{poly}]_{\mathrm{tot}}$ is the concentration of polymer, n is the number of binding sites on a polymer chain, K and α are adjustable parameters, K being the index of affinity and α the cooperativity index ($\alpha > 1$ for cooperative binding). The regular mixing model (Couderc *et al.*, 2001), which has been applied successfully to model mixtures of surfactants, ascribes to an enthalpy of mixing the difference between the experimental free energy of binding and the free energy of ideal mixing (i.e. mixing with zero enthalpy and only translational and rotational entropy of mixing). The regular mixing model constraints the activity of unbound surfactant and predicts:

$$[\text{surfactant}]_{free} = AX_s e^{\beta(1-X_s)^2} \qquad \textbf{(3)}$$

where X_s is the molar fraction of OTG in mixed micelles, and A and β are adjustable parameters. The interaction parameter β reflects the excess molar enthalpy due to the mixing of OTG micelles with polymer micelles (normalized to kT) (Couderc *et al.*, 2001; Peyre, 2002). If the regular mixing model is valid over the whole range of micelle compositions, including pure OTG micelles ($X_s = 1$), it is expected that A equals the cmc of OTG. Since we are also interested in the insertion of a few OTG molecules into polymer micelles, we will use A as a floating parameter that, therefore, corresponds to the equilibrium constant of OTG transfer from water into polymer micelles. Note that to determine X_s, one has to know the molar fraction of polymeric hydrophobic tails in a micelle. Assuming that all the polymer exists in the form of micelles ($\mathrm{cmc_{poly}} << 1$g/L) and that each *n*-octyl side group of the chain accounts for one independent tail, we can calculate Cp, the equivalent concentration of polymeric surfactants as Cp=[polymer]/$\mathrm{MW_{unit}}$, where the $\mathrm{MW_{unit}}$ is the molecular weight per octyl group in the polymer chain, namely 495, 1026, and 941 g/mol for A8-35, C45-68, and C22-43, respectively. It follows that

$$X_s = \frac{[OTG]_{bound}}{Cp + [OTG]_{bound}} \qquad \textbf{(4)}$$

The parameters of eqs 2 and 3 were fitted to the data (Figure 3.6b, c, Table 3.2). The parameter n should be constrained by the saturation of polymer. A complex comprising one polymer chain and one surfactant micelle (MW 44 000 g/mol) (Molina-Bolivar *et al.*, 2006) corresponds to n= ~4-5 for A8-35 or n= ~2 for C22-43.

Table 3.2. Parameters used to fit equations 3, 4 and 8 to experimental data from figures 3.3, 3.4, 3.6 and 3.7.

Surfactant/ polymer system	$\Delta H_{mic,s}$ (cal/mol)	β	A (mM)
OTG/A8-35	-	0.1-0.5 ^{ia}	15 ± 1 ^{i}
	-	0.8-1.2 ^{ii}	12.0 ± 1 ^{ii}
	-1350	0	11 ± 1
OTG/C22-43		1.2-1.5^{i}	11.5 ± 0.5 ^{i}
		0^{ii}	19 ± 2 ^{ii}
	-1350	0	13 ± 1
OTG/C45-68	-1350	0.5 ± 0.1	13 ± 1
C8E4/A8-35	-4700	0.3 ± 0.1	12 ± 1
C8E4/C22-43	-4700	0.4 ± 0.2	13 ± 1
DM/C22-43	-350	1 ± 0.1	0.23 ± 0.02 ^{b}

[a] Fits of isotherms in Figure 3.6b, c using eq 3 are indicated with the superscripts i and ii. One pair of parameters was adjusted to the entire range of $[\text{OTG}]_{\text{free}}$ (i) and the second pair of parameters was adjusted only to data with $[\text{OTG}]_{\text{free}} < 9$ mM (ii).

[b] Parameters used to fit the enthalpy in Figure 3.7b, with the corresponding $[\text{DM}]_{\text{free}}$ obtained using eq 3 and β=0 (see text).

Experimentally, a maximum of n = 2g/g is obtained for C22-43 (Figure 3.6c). In practice, the effect of the parameter n on the quality of fits in the range of accurate experimental measurements ($[OTG]_{free} < 9$mM) was not significant, and we fixed n=4 for simplicity. In the cooperative region, the regular mixing model gives a better fit of the data, but both models predict similar variations below ~ 9 mM OTG_{free}. Increasing the index of cooperativity (α in eq 2) does not improve the matching to data of the Hill model. Eq 2 rapidly predicts too low binding at low OTG (See Figure 3.6c to compare α=3 and α=6). Because of the large experimental uncertainty of data collected in the concentration range beyond the onset of the abrupt jump of binding -and in view of the turbidity noted in the case of A8-35/OTG mixed samples in this OTG concentration domain- the data points in this region suffer from a large experimental error on $[OTG]_{bound}$. Thus, we used measurements in the low $[OTG]_{free}$ concentration domain, and in this concentration range, several pairs of the parameters (A and β) and (K and α) adequately fit the data. For eq 3, the corresponding ranges of parameters are listed in Table 3.2. Interestingly, we obtained A values close to the actual cmc of OTG, making eq 3 valid with the same parameter both at low X_s and at $X_s = \sim 1$ (in the absence of polymer, the equilibrium between bound OTG and free OTG obviously obeys eq 3 with A=cmc). Since A always takes a value slightly larger than the actual cmc and given that β values are positive, it appears that the equilibrium between free and bound OTG is somewhat shifted toward the release of OTG in water, as compared to the ideal mixing scheme ($\beta = 0$, A = cmc). For $[OTG]_{free} < 9$ mM (i.e, polymer/OTG below 1 g/g for A8-35 (0.25 g/g for C22-43)), the molar fractions X_s

take values in the range of 0-0.62 (A8-35) or 0-0.47 (C22-43). In samples of maximal experimental concentration (and correspondingly OTGbound/polymer = ~2 g/g), X_s reaches values of 0.76 (A8-35) or 0.87 (C22-43).

3.6. Discussion

3.6.1. Thermodynamics of amphipol/detergent systems

The regular solution model has been applied previously to systems consisting of hydrophobically modified polymer/surfactant systems, but only to predict cmc or cic values (Bromberg *et al.*, 2000; Couderc *et al.*, 2001). The original model uses a βkT term to calculate the excess, as compared to ideality, of the free energy of interaction between two surfactants S1 and S2. We show (Supporting Information or Appendix B, equation S2) that the parameter β can be introduced in the expression of the enthalpy to calculate the heat of amphipol/surfactant mixing. The following relationship between the measured enthalpy (ΔH_{exp}) and the enthalpy of surfactant micellization (ΔH_{mic}) was derived, assuming that β is independent of the micelle composition and that all the polymer chains form assemblies (see Supporting Information or Appendix B for the detailed derivation of this equation)

$$N_{s,tot}\Delta H_{\text{exp}} \sim (N_s - N_{mic,s})\Delta H_{mic.s} + \frac{N_p N_s \beta RT}{N_s + N_p} \qquad \textbf{(5)}$$

where $N_{s,tot}$ and $N_{mic,s}$ are the molar amounts of total surfactant injected and of surfactant injected in micellar form, respectively, N_s is the molar amount of surfactant molecules that enter into associates, N_p is the total molar amount of alkyl groups linked to the amphipols, $X_s=N_s/(N_p+N_s)$ and $X_p=N_p/(N_s+N_p)$. The value of $N_{mic,s}$ depends only on known experimental parameters: the cmc, the surfactant concentration in the syringe, and the injected volume. Using eq 3, we obtain N_s and $N_{s.tot}$ as a function of A, β, X_s, and Cp, implementing an iterative calculation to account for dilution due to successive injections of the surfactant. Fits of the data with β (-10 to +10) and with $\Delta H_{s,\,mic}$ are plotted in Figure 3.7, within the uncertainty (ca. 10%) of the measured enthalpy of micellization The corresponding parameters are listed in Table 3.2. Examples of the influence of each parameter on the fit are provided in Figure 3.2S (Supporting Information or Appendix B). The regular solution approximation gave satisfactory fits to the experimental data (Figure 3.7) for each polymer/surfactant systems tested experimentally (Figures 3.3 and 3.4). Relative differences between measured and calculated enthalpies were less than 10 % for systems of polymer concentrations of 2.5 and 5 g/L (OTG or C8E4) or 0.1-1g/L (DM/C22-43). For systems of the highest polymer concentrations, the fits were excellent over a large range of surfactant final concentrations (0-15 mM for OTG and C8E4, 0- 0.3 mM for DM), independently of the surfactant concentration in the titrant ([OTG]: 100 or 13.5 mM). The corresponding X_s values for the mixed micelles are in the following ranges: 0-0.62 (OTG/A8-35, OTG/C22-43, and C8E4/A8-35), 0-0.54 (C8E4/C22-43 at 5g/L), or 0- 0.55 (DM/C22-43).

The regular mixing scheme provides a simple framework to interpret the heat evolved upon dilution of detergent into an amphipol solution using only two parameters (A and β).

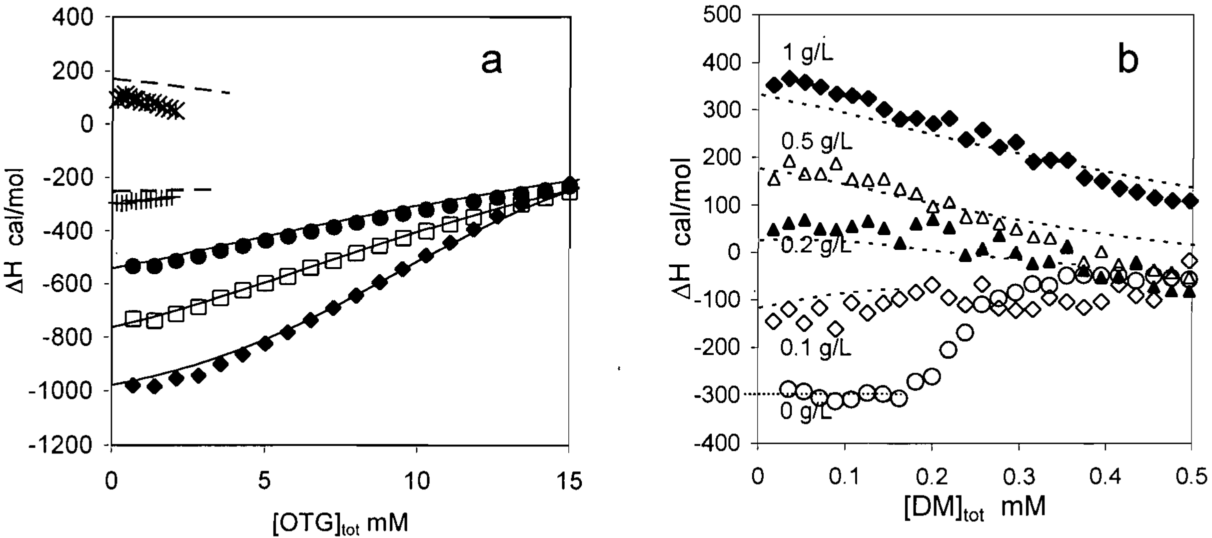

Figure 3.7: Plots of the changes of the enthalpy of dilution of a detergent solution in amphipol solutions as a function of total detergent concentration in the cell. (a) Titrations with 100 mM OTG in solutions of A8-35 (◆) 1.0, (□) 2.5, or (●) 5.0 g/L or 13.5 mM OTG in A8-35 (+) 1.0 g/L or (×) 5.0 g/L. (b) Titration with 2.5 mM DM in C22-43 (polymer concentrations as quoted in figure). The lines (solid or dashed) correspond to fits to the regular mixing model (eqs 3-5) with parameters listed in Table 3.2. Some of the experimental data are taken from Figures 3.3 and 3.4, and additional data are plotted (titrations of a 13.5 mM OTG solution in solutions of A8-35 and 2.5 mM DM in (◊) 0.1 g/L C22-43 and (Δ) 0.5 g/L C22-43).

The agreement of the model with the experimental data extends over a range of compositions of the amphipol/detergent mixed micelles, starting with various concentrations of either surfactant or amphipol. It is interesting to note that the value of β (0-1) giving the best fits implies that the deviation from ideal mixing is weak and that the enthalpy of transfer of a detergent molecule from a detergent micelle to an amphipol micelle is almost zero. This result was not anticipated, and most probably is not a general phenomenon. The contribution of nonideal mixing terms may be much higher for amphipol/detergent systems able to undergo specific interactions, such as hydrogen bonding. The endothermic contribution observed upon dilution of micellar surfactant solutions in amphipol solutions, as compared to their dilution in buffer, reflects the fact that a fraction of the demicellized surfactants are transferred from water to the micelle-like environment provided by the amphipols.

In the case of DM, it turns out that the enthalpy of micellization of DM (+350 cal/mol) is of the same order as the correction due to nonideality (the term $N_pN_s/(N_p+N_s)\beta RT$ in eq 5). In this case, the contribution of nonideality cannot be neglected. We used the value $\beta = 1$ (Table 3.2) to obtain a good fit to the experimental enthalpies for low [DM]. This value allows one to fit the change from exothermic to endothermic contributions with increasing polymer concentration. The value of β affects slightly the slope of the thermograms in the high DM concentration range. We obtained an excellent fit of the experimental data assuming (i) ideal partition of DM between polymer micelles and water ($\beta = 0$ in eq 3) and (ii) $\beta = 1$ in eq 5. If one selects $\beta = 1$ in eq 3, which would account for regular, nonideal partition, the fits display a weaker (negative) slope that suggests that the regular mixing is not valid in this case and that a weak excess entropy of mixing term should exist. The difference in hydrophobic tail (n-dodecyl for DM vs n-octyl in the amphipols) may lead to some entropic contribution to the mixing as a consequence of the mismatch among the hydrophobes. The analysis of ITC titrations with DM points nevertheless to the weak endothermic contribution to mixing, quite similar to the results obtained with the detergents having *n*-octyl tails.

In the case of the OTG/amphipol systems, we can compare the parameters obtained by fitting ITC curves to the parameters obtained independently of the binding isotherms and equation 3 (Figures 3.6b, c, Table 3.2). The remarkable similarity of the two sets of values lends further support to the fact that the essential features of the energy of mixing can be derived accurately from a thermodynamic analysis of the mixing. Thus eq 3 is not only an interesting parametrization of isotherms but adds to the conventional phenomenological expressions of cooperativity (e.g., eq 2) the interesting possibility to estimate an enthalpic contribution of mixing detergent with polymer micelles.

3.6.2. Size and structure of amphipol/detergents mixed micelles

Throughout the analysis, we assumed that the hydrophobic tails of the amphipols do not escape the micelles or the mixed amphipol/surfactant micelles over the whole range of sample compositions. The analysis of enthalpies using the regular mixing theory does not refer to any other constraint in the conformation of the polymer chains. In the case of protein/surfactant mixtures, cooperative binding also takes place, but the cooperative nature of surfactant binding onto proteins, especially in the case of ionic surfactants, is ascribed to the unfolding of hydrophobic peptidic residues that become exposed to the hydrophilic environment (Robinson & Tanford, 1975; Goddard & Ananthapadmanabhan, 1992; La Mesa, 2005). The cooperative association of surfactant is accompanied, in this case, by a marked increase of the radius of the polypeptide chain (Goddard & Ananthapadmanabhan, 1992; La Mesa, 2005). In contrast, in all the systems we examined, except OTG/A8-35, the mixed micelles do not grow in size significantly upon association, revealing significant differences in cooperative assembly of neutral detergent/amphipols and protein/surfactant systems.

The special case of the OTG/A8-35 system merits further discussion, since it is currently used in protocols for the purification and isolation of integral membrane protein. The experimental enthalpy data for this system were fitted suitably via the general analysis presented; however, unlike all the other systems, OTG/A8-35 mixed micelles grow markedly in size (DLS data) and molecular weight (scattered light intensity data) with increasing OTG concentration and the OTG/A8-35 complexes become insoluble in water above a threshold OTG concentration. We suggest that this behavior is primarily a consequence of the unusual properties of the OTG micelles. Unlike DM or C8E4, which form spherical micelles, OTG forms elongated micelles that tend to grow in size with increasing OTG concentration (see ref (Molina-Bolivar *et al.*, 2006) and Figure 3.1S, Supporting Information or Appendix B). The solubility in water of OTG micelles and of OTG/A8-35 may be more sensitive to modifications of their composition, as compared to smaller micelles, due to the higher translational entropy of smaller objects. Further studies are needed in order to understand why only the OTG/A8-35 exhibits this peculiar behavior and why OTG/C22-43 mixed micelles, for instance, show no propensity toward growth and phase separation.

3.6.3. Guidelines for the applications of amphipol/detergent systems in biology

Several issues need to be addressed concerning proper handling of amphipol/detergent micelles in biology. Of primary importance for structural analysis of proteins trapped in amphipol/detergent mixtures is the fact that the mixed micelles need to remain small and monodisperse in size and that the composition of the mixed micelle during all the steps of IMP purification is known and, ideally, controlled. We observed that, except in the case of OTG/A8-35, the mixed detergent amphipol micelles remained small (< 5 nm), independent of detergent concentration, and they did not form objects polydispersed in size. It is interesting to note that these features are shared by two amphipols with totally different chemistry: A8-35, an anionic polymer, and C22-43, a cationic polymer. The two polymers, however, share common structural features: low molecular weight and similar molar ratios of the neutral, charged, and hydrophobic units. Deviation from this optimal composition and chain length affects dramatically the polydispersity of polymer assemblies as shown with C45-68, independently of the chemistry of the polymers.

The composition of amphipol/detergent mixed micelles and the free detergent concentration in solution can be estimated from eq 3, using $A\sim$ cmc of the detergent and $0 < \beta < 1$. In systems of low total concentration of detergent (<< cmc), the molar fraction of detergent in mixed micelles is very low, and the micelles are essentially composed of amphipol. Another conclusion of the analysis is that when the fraction (X_S) of detergent in mixed micelles is large, the concentration of free detergent in solution must approach the cmc of the pure detergent. A consequence of this behavior is that the composition of mixed micelles is controlled by the molar ratio of total detergent/amphipol alkyl groups in solution. Two cases are important from a practical viewpoint: (i) low value of this ratio, for the removal of detergent from amphipol micelles (protein trapping in amphipol), and (ii) large excess of detergent/amphipol alkyl groups, for the reconstitution of proteins in detergent or in lipid vesicles. In case i, eqs 3 and 4 show that in solutions of surfactant concentrations well below the cmc, the mol % of amphipol-bound detergent is of the order of $[\text{detergent}]_{\text{total}}$/(cmc + [amphipol tails]), in equilibrium with a concentration of free detergent = ~ cmc.X_S. Therefore, the proportion of detergent in mixed micelles decreases gradually by decreasing the total detergent concentration in samples, although concentrations markedly below the cmc (< cmc/100) are required to achieve a negligible contribution of the detergent to the amphipol micelles (< 1 mol %). In contrast, under the conditions of amphipol detachment from proteins and reconstitution of the protein in detergent (case ii), excess detergent is used and the mixed amphipol/micelles are saturated with surfactant. In many respects, they behave as detergent micelles. Figure 3.6c suggests that the amount of detergent per amphipol may reach a maximum value (~2 g/g for OTG/C22-43). Under such conditions, mixed micelles of detergent/amphipol hydrophobes of molar ratio up to 80:20 will coexist with detergent micelles.

3.7. Conclusion

A consensus picture of amphipol/neutral surfactant complexes emerges on the basis of measurements of their size distributions, their average compositions, and their enthalpies of formation. Our results consistently point to the formation of mixed micelles with small sizes, in equilibrium with unbound surfactants (typical radii < 5 nm provided that the detergent forms approximately spherical micelles). The binding isotherms can be analyzed by the model of regular mixing between polymer micelles (initially present) and surfactant micelles. The behavior is generally close to ideal mixing, and the enthalpies of surfactants association into amphipols appear very similar to their enthalpies of self-micellization. Differences in the chemical structures of the polymers (anionic or cationic) and of the surfactants (PEO or sugar heads, C8 or C12 tails) may add subtle specificities, such as lower solubility of the complexes or weaker entropic terms, but they do not change significantly the overall properties of the system. The absence of any marked effect of the chemical nature of the amphiphiles must be seen as an indication that the association is mostly driven by the hydrophobic tails of surfactants seeking a micelle-like hydrophobic core, irrespective of the detailed nature of the hydrophilic corona.

When no specific interaction takes place between the micelles of amphipols and the surfactant, the regular mixing model provides a good prediction of the cooperativity of the binding close to the cmc of the surfactant. A critical interaction concentration (cic), however, does not emerge from this model, which assumes a continuous, although rapid, variation of the composition of the mixed micelles with surfactant concentration. As compared to other systems combining polymers and surfactants, the higher hydrophobicity of amphipols has the rather unique consequence that polymer micelles are present in solution over the entire experimental range of concentrations. It is likely that the absence of true cic is a consequence of the self-assembly of amphipols in absence of surfactants. No abrupt change of chain properties is expected when a surfactant enters in a micelle containing already several amphiphilic moieties.

Finally, our results show that the micelles of amphipols contain a significant fraction of neutral detergents, even below the cmc of the surfactant. This point is of prime importance when preparing polymer/protein complexes. It indicates for instance that a continuous perturbation of the micellar environment can be achieved by gradual changes of the concentrations of polymer and/or detergent. This observation emphasizes the need to rid mixed amphipol/protein samples of detergents and to use detergent concentrations well below cmc/10 to obtain complexes that are depleted of excess detergent. The sensitivity of some protein activity to trace amount of detergent additives may also originate from the good partitioning of surfactant in amphipol assemblies (Champeil *et al.*, 2000).

3.8. ACKNOWLEDGMENTS

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3.9. SUPPORTING INFORMATION (Appendix B)

Detailed description of synthesis and characterization of the amphipols C22-43 and C45-68. Derivations of equations S1, S2 and S3, an illustration of changes of the calculated enthalpies for various pairs of A and β, and a plot of the changes of the hydrodynamic radius of OTG micelles as a function of OTG concentration. This material is available free of charge via the Internet at http://pubs.acs.org (Appendix B).

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CHAPTER FOUR

RESEARCH PAPER

Enthalpies of Exchange between Detergents and Amphipols at the Surface of Integral Membrane Proteins

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4.1. Abstract

Amphiphilic macromolecules, known as amphipols, have emerged as promising candidates to replace conventional surfactants (esp. neutral detergents) for the solubilization and stabilization in water of highly hydrophobic integral membrane proteins. In practice, the protein/amphipol complexes are prepared by competitive exchange of a protein-bound detergent for the polymer, but little is known on the relative thermodynamic stability of the protein/poly(amphiphile) complexes. This prompted us to investigate the energy of formation and of exchange of the amphiphile in complexes between various detergents, amphipols and proteins (a porine -OmpA- and bacteriorhodopsin -BR-). The proteins were initially prepared as complexes in detergents (OTG or C8E4) or associated with two amphipols of different hydrophilic moieties: a carboxylate-based amphipol, and a phosphorylcholine-based amphipol. The heats evolved upon exchange of detergent for amphipol, and amphipol for detergent have been studied by isothermal titration calorimetry. Gradual and abrupt exchanges were performed by slow addition of detergent or rapid dilution in excess detergent of the protein/amphipol complexes. The energy of the conversion of protein/detergent complexes into protein/amphipol complexes is of the order of magnitude of the energy of demicellization, whereas conversion of the protein/amphipol complexes into protein/detergent complexes is almost athermal. A model of regular mixing is shown to be applicable to protein complexes. For both amphipols, the exchanges with detergents onto protein complexes appear to be essentially similar to the processus of mixed micellization.

4.2. Keywords

Amphipols; Detergent; Mixed micelles; Integral membrane proteins; ITC; Amphiphilic macromolecules.

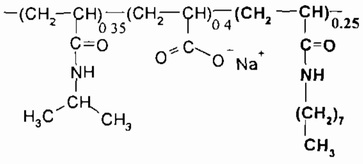
4.3. Introduction

Studies of the chemistry and biology of membrane proteins (MP), which are of critical importance in the biology of cells and the therapeutic action of most current drugs, are hampered by experimental difficulties related to the inherent insolubility in water of this class of proteins. To analyze the structure and functions of MPs by standard tools one needs to coerce them into water by entrapment into micellar systems. A number of mild detergents have been found adequate to solubilize most MPs by mimicking the natural lipid bilayer environment (Helenius & Simons, 1975; le Maire *et al.*, 2000; Garavito & Ferguson-Miller, 2001; McGregor *et al.*, 2003; Anatrace). Albeit detergent/MP complexes are fragile entities and show a propensity towards fatal aggregation upon dilution or prolonged storage (Breyton *et al.*, 1997; Zhou *et al.*, 2001; le Maire *et al.*, 2006). In addition, evidence accumulates that the conformation of some membrane proteins is altered upon interaction with detergents. Some shortcomings of detergents can be alleviated through the use of amphipathic polymers (reviewed in refs Popot *et al.*, 2003; Sanders *et al.*, 2004), APols, that consist of a hydrophilic polymeric backbone grafted with alkyl chains (Tribet *et al.*, 1996; Diab *et al.*, 2007). Like detergents, APols bind to the transmembrane domain of MPs, presumably in a way such that the hydrophobic groups of the polymer interact with the hydrophobic surface of the protein while the polar groups provide a hydrophilic corona thereby maintaining the colloidal stability of the MP/amphipol complex. Spontaneous desorption of APols from the protein is unlikely to occur, since each APol chain has multiple points of attachment to the hydrophobic surface of the protein. Current APol design guidelines derive from empirical rules and laboratory "know-how". The robustness of the structural guidelines would be greatly enhanced by an understanding of the factors contributing to the thermodynamic stability of APol/MP complexes. There is surprisingly little data available on the thermodynamics of the APol/MP complexation and on whether the APol attachment is entropic or enthalpic in nature. The large range of free energy of micellization encountered in surfactant systems (Zana, 1996) suggests, by analogy, that APol self-association and binding onto proteins will depend on the nature of the hydrophilic moieties in the chains, yet there is no evidence to support this hypothesis.

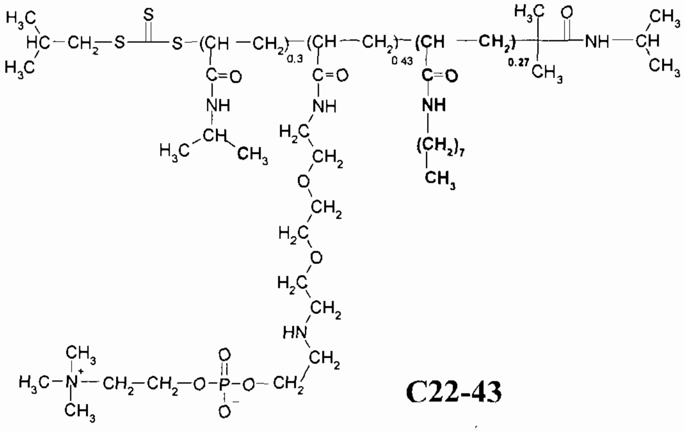
We had in hand two APols of similar hydrophilic/lipophilic balance but different chemical composition: a polyanion (A-8-35) (Tribet *et al.*, 1996), which is a copolymer of *N*-n-octylacrylamide, *N*-isopropylacrylamide, and sodium acrylate, and a polybetaine (C22-43) (Diab *et al.*, 2007), which consists of *N*-n-octylacrylamide, *N*-isopropylacrylamide, and a phosphorylcholine (PC)-substituted acrylamide (Figure 4.1 and table 4.1). Although different in chemical structure, both polymers are equally effective in solubilizing MPs, such as bacteriorhodopsin (BR), and protecting them against denaturation in neutral aqueous environment. Differences in the stability of the two types of APol/MP complexes become apparent only under conditions of stress, such as acidic pH or high salinity: the

APol/protein complexes formed with the PC-based polymer exhibits enhanced stability in the presence of salts and in acidic pH environment, compared to the complexes formed by the acrylate-based APols, as expected on the basis of the different chemical structure of the hydrophilic groups exposed to the aqueous medium (Lowe & McCormick, 2002; Diab *et al.*, 2007).

Using these two APols, we examine here the thermodynamics associated with each step of the solubilization by APols of two MPs: the bacteriorhodopsin from Halobacterium salinarium (BR) and the porine OmpA, which are representative of two classes of MPs: BR is a 7 α-helices bundle whereas OmpA is a β-barrel. Using isothermal titration calorimetry (ITC), we monitored the trapping of MP within APol starting from detergent/MP complexes and the reverse process, namely the exchange of APol for detergent, starting with APol/MP complexes devoid of residual detergent. We found that the enthalpy of MP trapping by either APol is remarkably high, reaching values on the order of the enthalpy of demicellization of the detergent, whereas the formation of detergent/MP complexes from APol/MP complexes is quasi-athermal. The data were analyzed using the regular mixing model, which provided a suitable framework for the interpretation of the binding energetics, underlining the similarities of the APol-binding process with the binding of conventional detergents.



A8-35



C22-43

Figure 4.1: Chemical structure of the amphipols employed.

Table 4.1. Characteristics of the polymers employed in the study.

	Composition (mol %)					
Polymer	NIPAM	AA[a]	PC	C_8H_{17}	M_n (g/mol)	M_w/M_n
A8-35	40[b]	35[b]		25[b]	7500	1.6
C22-43	30[c]	--	43[c]	27[c]	22000[c]	1.2

[a] AA: acrylic acid; NIPAM: *N*-isopropylacrylamide; PC: *N*-phosphorylcholine-*N*-[(ethylenedioxybis(ethyl) acrylamide]; and C_8H_{17}: *n*-octylacrylamide.

[b] See refs (Tribet *et al.*, 1997; Gohon *et al.*, 2006).

[c] See Supporting Information (Appendix B).

4.4. Materials and methods

n-Octylthioglucoside (OTG, 98%), and *n*-octyl-tetraethyleneglycol (C8E4) were purchased from Sigma Chemicals and used without further purification. The amphipols, A8-35, FApol (a fluorescence dye (<1%) were attached to the A8-35 to adjust the concentration of polymers in solutions with or without the protein following the absorbance at 476nm) and C22-43, were synthesized as described elsewhere (Tribet *et al.,* 1996; Diab *et al.,* 2007; Zoonens *et al.,* 2007). The MPs, tOmpA and bacteriorhodopsin (BR), were isolated from *E. coli* and from the plasma membrane of *Halobacterium salinarium* (strain S9), respectively, as described previously (Zoonens, 2004; Diab *et al.*, 2007). They were obtained as detergent complexes (C8E4/tOmpA in Tris buffer and OTG/BR in phosphate buffer). The MPs concentration in solutions for analysis was determined from the absorbance at 554 nm (ε=1.60 L g^{-1} cm^{-1}) and 280 nm (ε=2.2.10^3 L $g^{-1}cm^{-1}$) for BR and tOmpA respectively using a UV-visible Hewlett-Packard 8453 diode array spectrometer.

4.4.1. Preparation of C8E4/tOmpA complexes

They were prepared as previously described (Zoonens *et al.*, 2005).

4.4.2. Preparation of Apol/tOmpA complexes

A sample of tOmpA in C8E4 (2 ml, 2 g L^{-1}) is supplemented with amphipols (A8-35 or C22-43) in a 1:4 (w/w) protein/amphipol ratio. After 15 minutes of incubation, the samples solutions were diluted below the critical micelle concentration of the detergent (cmc) and then the detergent monomers were eliminated by incubation for 2 hours on polystyrene beads (Bio-Beads SM2). The beads were removed by a short centrifugation. The resulting solution was dialyzed against the same buffer (Tris/HCl 20 mM, pH 8.0, 100 mM NaCl).

4.4.3. Preparation of OTG/BR complexes

Purple membrane from H. Salinarium (strain S9) was solubilized from 100 mM OTG in 20 mM sodium phosphate buffer (pH 6.8). Soluble OTG/BR complexes were recovered from the supernatant after centrifugation for 30 minutes at 200,000xg in the TLA 100 rotor of a Beckman TL100 centrifuge. The supernatant was layered onto 4.5 ml of 5-20% (wt/wt) linear sucrose density gradient in the same buffer containing 15 mM of OTG. The tubes were centrifuged at 4 ^{0}C for 18 h at 37 000 rpm in an MLS50 rotor (~ 150,000xg). The resulting OTG/BR band was taken and dialyzed against phosphate buffer 20mM, pH 7.0, OTG 15 mM for 24 hours (the dialysis media was changed 2-3 times) to eliminate the sucrose and to equilibrate the concentrations of detergent and salt in the protein sample. The sample is to be injected in solution of A8-35 and C22-43 to evaluate the enthalpy of exchange of OTG into A8-35 and C22-43 on the surface of BR partially depleted from lipids. The concentration and the quality of BR were evaluated at each step from the UV-vis absorbance at 554nm (corresponding to the native BR) and 320nm (corresponding to retinal released upon BR denaturation).

4.4.4. Amphipols solutions preparation

Stock polymer solutions (2 to 20 g/L) were prepared by dissolving freeze-dried samples in deionised water under gentle stirring for at least 2 h at room temperature. The amphipol solution in water was supplemented with concentrated Tris buffer (pH 8.0) to achieve a final concentration of 20 mM and 100 mM NaCl or with a phosphate buffer (pH 7.0) to achieve a final concentration of 20 mM and 100 mM NaCl. The resulting solutions were dialyzed against the same buffer as the detergent/MPs complexes solutions used for analysis to ensure that they have the same ionic concentration.

4.4.5. Isothermal titration calorimetry experiments

The calorimetric measurements were performed with a Microcal VP-ITC having a cell volume of 1.43 mL and fitted with a 300-μL syringe continuously stirred (300 rpm) during the titration. All measurements were performed at 25 °C. The reference cell was filled with the buffer used for the samples preparation. Data were analyzed using the Microcal ORIGIN software. The experimental enthalpy change h_i resulting from the *i-th* solution injection was obtained by integration of the raw data signal. The integrated molar enthalpy change per injection (ΔH_i, in J/mol) was obtained by dividing h_i by the number of moles of injectant added, n_i; hence $\Delta H_i = h_i/n_i$, resulting in enthalpograms, which are plots of ΔH_i as a function of surfactant or IMP concentrations in the calorimeter sample cell.

4.4.6. Amphipol binding to MPs solubilized in detergent

With tOmpA: A solution of the polymer (1.0 or 5.0 g L^{-1}) in Tris buffer was placed in the sample cell. Aliquots (8 μL) of a solution of C8E4/tOmpA in Tris buffer (tOmpA: 1.0 or 2.0 g L^{-1}, C8E4: 10 mM) were injected into the sample cell at intervals of 300 s. Two control experiments were performed that involved injection of C8E4 (10 mM) (i) in Tris buffer and (ii) in a solution of the polymers (A8-35 or C22-43, 1.0 and 5.0 g L^{-1}) in Tris buffer.

With BR: A solution of the polymer (1.0 or 5.0 g L^{-1}) in phosphate buffer was placed in the sample cell. Aliquots (8 μL) of a solution of OTG/BR in phosphate buffer (BR: 0.5 or 1.0 g L^{-1}, OTG: 15 mM) were injected into the sample cell at intervals of 300 s. Two control experiments were performed that involved injection of OTG 15 mM (i) in phosphate buffer and (ii) in a solution of the polymers (A8-35 or C22-43, 1.0 and 5.0 g L^{-1}) in phosphate buffer.

4.4.7. Reconstitution of detergent/MP complexes from MP/Apol complexes by slow exchange

A solution of Apol/MP (FApol/tOmpA, or C22-43/tOmpA, tOmpA concentration: 0.5 or 0.25 g L^{-1}, polymer concentration 2 or 1 g L^{-1} respectively) in Tris buffer was placed in the sample cell. Aliquots (2 μL) of a solution of C8E4 (100 mM) in Tris buffer were injected into the sample cell at intervals of 300 s (Figure 4.4). Control experiments involving the titration of a solution of C8E4 (100 mM) in Tris buffer into Tris buffer and into polymers in Tris buffer (FApol and C22-43, concentration: 2 or 1 g L^{-1}) were performed under identical conditions (Figure 4.4). The concentration of the FApol in protein-free solutions was adjusted to the concentration of FApol in complexes solutions using the FApol absorbance at 476 nm.

4.4.8. Reconstitution of detergent/MP complexes from FApol/MP complexes by fast exchange

A solution of C8E4 (20 mM) in Tris buffer was placed in the sample cell. Aliquots (8 μL) of a solution of A8-35/tOmpA (tOmpA: 1 g L^{-1} or 2 g L^{-1}, A8-35: 4 g L^{-1} or 8 g L^{-1}) or C22-43/tOmpA (tOmpA: 1 g L^{-1} or 2 g L^{-1}, C22-43: 4.4 g L^{-1} or 8.8 g L^{-1}) were injected in the sample cell at intervals of 300 s (Figure 4.5 A, B, C, E). Control experiments were performed by adding to a solution of C8E4 (20mM) in Tris buffer (i) Tris buffer, (ii) A8-35 in Tris buffer (4 g L^{-1} or 8 g L^{-1}) and (ii) C22-43 in Tris buffer (4 g L^{-1} or 8 g L^{-1}) (Figure 4.5 D, F).

4.5. Results

4.5.1. Enthalpy of formation of APol/MP complexes

Aliquots of a solution of either C8E4/tOmpA in Tris buffer of pH 8.0 or OTG/BR in phosphate buffer of pH 7.0 were titrated at constant temperature (25 ^{0}C) into amphipols solutions (A8-35 and C22-43, 1 and 5 g L^{-1}) in the same buffer. C8E4 and OTG solutions in the absence of proteins were injected at the same concentration (as in complexes solutions) into buffer and amphipols solutions (A8-35 and C22-43, 1 and 5 g L^{-1}). After each injection, heat was released as a result of the various processes occurring in the solution. In all experiments, the raw data recorded show measurable heat with precision and without any interference or noise (Figure 4.2 A, B). The enthalpy profiles obtained from the integration of each individual peak for different concentrations range of amphipols and proteins, for different types of proteins and amphipols are presented in figure 4.2 (Figure 4.2 C, D, E, F). The heat evolved upon injection of the complexes into APols solutions was constant, and independent of the number of injection for all concentrations of proteins tested, but it depended significantly on the amphipol concentration (Figure 4.2 D, F), and the presence or absence of proteins (Figure 4.2 C, D, E, F).

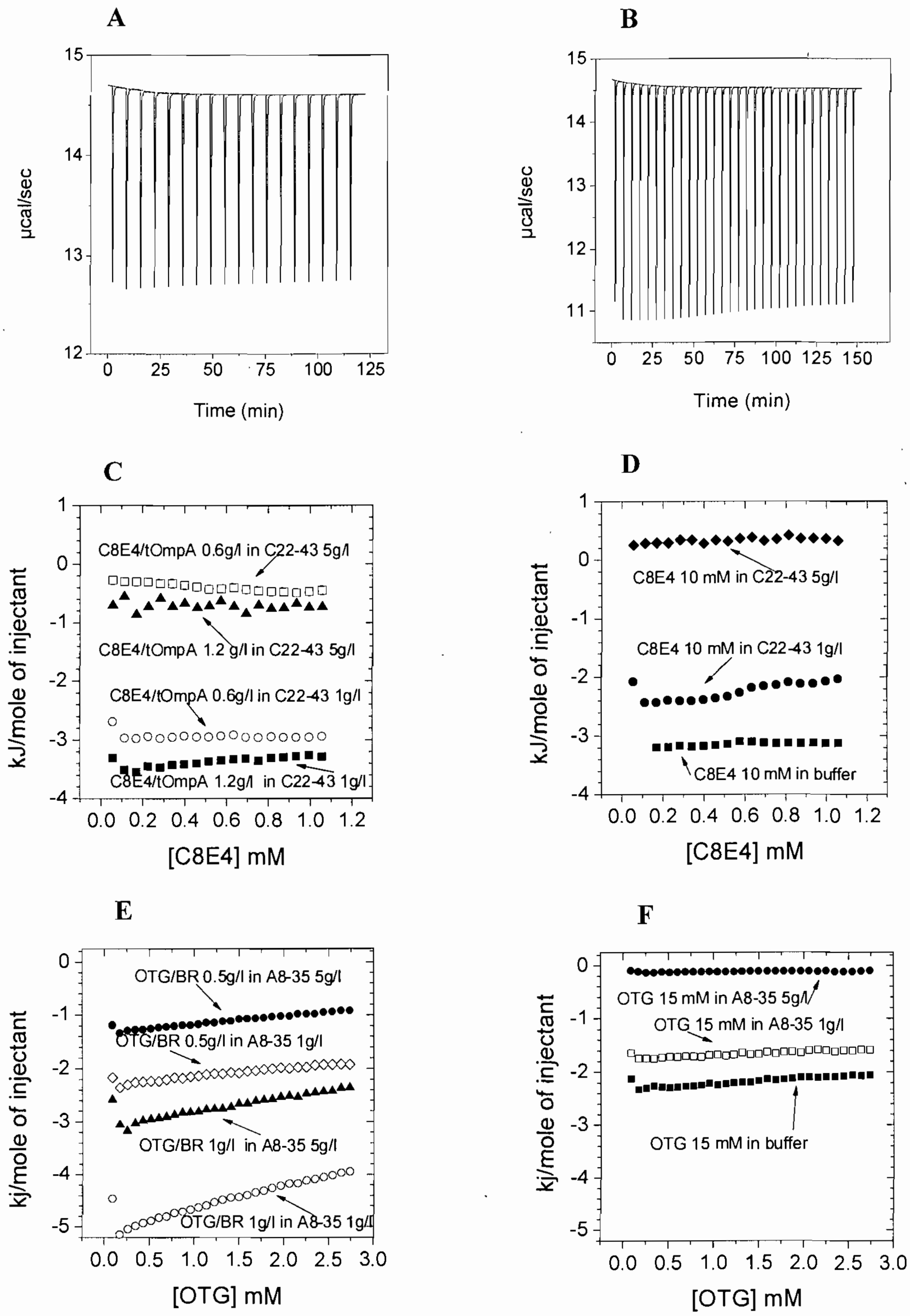
In the presence of polymers, the heat evolved upon injecting a micellar solution of surfactant (without MP) reflects not only the heat of demicellization but also the enthalpy of interaction between the polymer and the surfactant. The heat evolved upon surfactant injection into polymer solution was significantly less than in the absence of polymer, over the whole range of surfactant concentrations (Figure 4.2 D, F, and Table 4.2), pointing to an endothermic contribution due the energy of binding of the surfactant onto polymer (Figure 4.2 D, F). The magnitude of the effect increases when increasing polymer concentration (Figure 4.2 D, F, and Table 4.2), implying that the surfactant binding occurs in proportion to the amount of polymer chains in solution. A quantitative analysis of binding in term of molar binding degree was reported previously (Diab *et al.*, 2007).

The heat of dilution of surfactants alone in amphipols solutions was obtained from the enthalpograms (Table 4.2, ΔH_{exp}). It can be calculated also using the regular mixing model (using eq1 and eq2, $\beta = 1.1$, ΔH_{mic}= -16720 J/mol or -5434 J/mol for C8E4 and OTG respectively, and CMC = 8.2 mM and 9 mM for C8E4 and OTG respectively) as previously reported (Diab *et al*, 2007) (Table 4.2, $\Delta H_{calculated}$):

$$\Delta H_{measured} = \Delta H_{mic}([\text{detergent}]_{syringe} - \text{CMC})/[\text{detergent}]_{syringe} + Q_{mixed}/N_{tot}) \quad \mathbf{(1)}$$

Our data show similarity between the measured enthalpies and the calculated enthalpies (Table 4.2).

The heat released for the dilution of detergent/IMP complexes in amphipols solutions (Figure 4.2, C, E, and scheme 4.1) reflects many processes: (i) the demicellization of the detergent micelles not associated to the MP (free detergent), (ii) the desorption of the detergent from the detergent/MP complexes, (iii) the association of polymers and detergents into mixed micelles, and (iv) the formation of amphipol/MP complexes.



Caption on page 228

Figure 4.2: Enthalpy of dilution (Δ*H*) of C8E4 and OTG or C8E4/tOmpA and OTG/BR complexes into solutions of amphipols. All solutions were prepared in 100 mM NaCl, 20 mM Tris HCl buffer, pH 8.0 (in the case of OmpA) and in 100 mM NaCl, 20 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 6.8 (in the case of BR). (A) Raw data for the titration of C8E4/tOmpA (tOmpA 0.6 g L^{-1}, C8E4 10 mM) into C22-43 (1 g L^{-1}), and (B) OTG/BR (BR 0.5 g L^{-1}, OTG 15 mM) into A8-35 (1 g L^{-1}). (C) Injection of C8E4/tOmpA complexes (tOmpA 1.2 g L^{-1} in 10 mM C8E4) into 1 g L^{-1} C22-43 (■) or 5 g L^{-1} C22-43 (▲) and injection of C8E4/tOmpA (tOmpA 0.6 g L^{-1} in 10 mM C8E4) into 1 g L^{-1} C22-43 (○) or 5 g L^{-1} C22-43 (□), and (D) corresponding injection of C8E4 (10 mM) into solutions of 1 g L^{-1} C22-43 (●) or 5 g L^{-1} C22-43 (◆), and into Tris buffer (■). (E) Injection of OTG/BR complexes (BR 0.5 g L^{-1} in 15 mM OTG) into 1 g L^{-1} A8-35 (◇) or 5 g L^{-1} A8-35 (●) and injection of OTG/BR (BR 1 g L^{-1} in 15 mM OTG) into 1 g L^{-1} A8-35 (○) or into 5 g L^{-1} (▲), and (F) corresponding injection of OTG (15 mM) in buffer into solutions of 1 g L^{-1} A8-35 (□) or A8-35 5 g L^{-1} (●), and into phosphate buffer (■). In order to facilitate the comparison of the enthalpies in the presence and absence of BR and tOmpA, the *x*-axis refers to the concentration of OTG and C8E4 in the samples injected. Temperature: 25°C.

Table 4.2: Enthalpy of dilution (measured and calculated) of OTG 15 mM and C8E4 10 mM into buffer and buffer containing APols.

APols	ΔH_{exp} (KJ/mol)	ΔH_{calc} (KJ/mol)[a]
Dilution of OTG 15mM		
No APols	-2.20 ± 0.08	-2.17
A8-35 (1 g L^{-1})	-1.59	-1.60
A8-35 (5 g L^{-1})	-0.46	+0.046
C22-43 (1 g L^{-1})	-2.10	-1.84
C22-43 (5 g L^{-1})	-2.30	-0.765
Dilution of C8E4 10mM		
No APols	-3.20	-3.00
A8-35 (1 g L^{-1})	-1.70	-1.65
A8-35 (5 g L^{-1})	+2.32	+2.32
C22-43 (1 g L^{-1})	-2.38	-2.21
C22-43 (5 g L^{-1})	+0.33	+0.44

[a] Calculated from equation 2

Assuming that the enthalpies of dilution of detergents monomers and micelles are negligible (Diab *et al.*, 2007), the heat evolved upon dilution of MP/detergent complexes into amphipols solutions can be examined as follows and as presented in scheme 4.1:

✱ Q (exchange detergent-amphipol) = Q (desorption of the detergent) + Q (formation amphipol/MP complexes) (Values are presented in table 4.3).

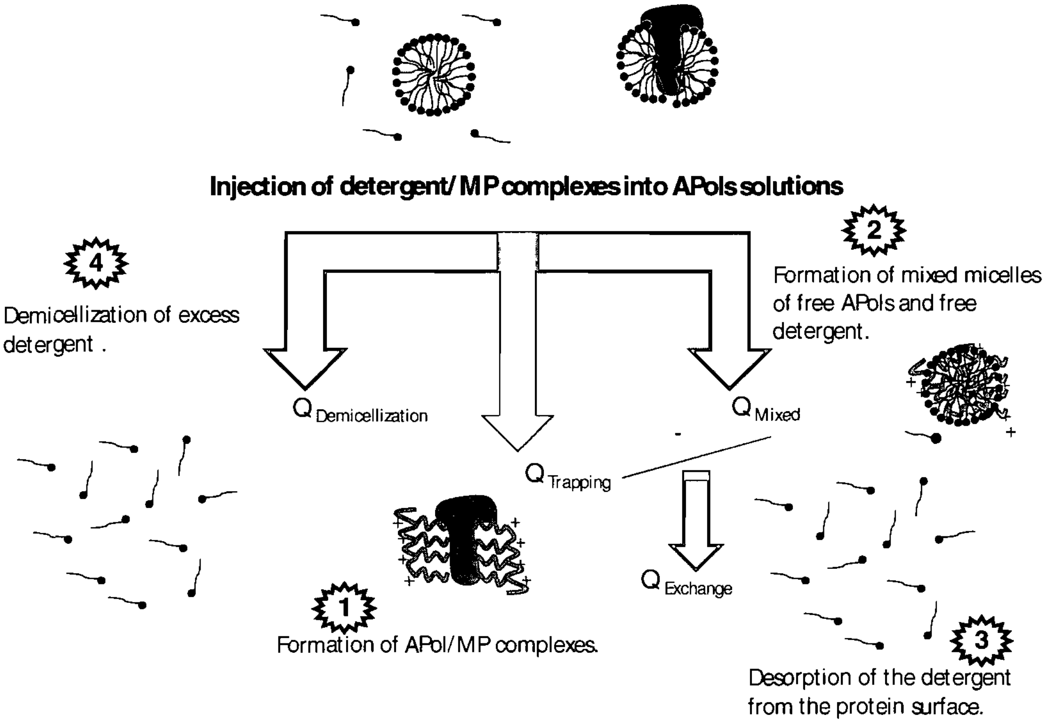
✱ Q (demicellization) = ΔH (demicellization) x (fraction of micelles in buffer) x (number of mole of unbound detergent injected).

✱ Q (mixed), which is the heat resulting from the associations between amphipols and detergents can be calculated as described elsewhere (Diab *et al.*, 2007) and can be obtained from the data obtained from the injection of detergent in the absence of proteins into amphipols solutions. The heat of the association of amphipols/detergents can be written:

$$Q_{\text{mixed}} \times (N_{tot}) = Ns\ \Delta H_{mic} + \beta.R.T.N_p.N_s/(N_p+N_s) \qquad \textbf{(2)}$$

where N_{tot} is the total number of added detergent, Ns is the number of moles of detergent that form complexes with amphipols ($N_s=N_p.X_s/(1-X_s)$), N_p is the number of mol C_8 of amphipol, ΔH_{mic} is the enthalpy of micellization of the detergent, and β is an adjustable parameter (non-ideality term).

The energetics of the formation of MP/amphipols complexes were evaluated from the data obtained (Table 4.3). The heat evolved upon dilution of MP/detergents complexes into amphipols solutions (Figure 4.2 C, E) was significantly higher than the heat evolved upon addition micellar solution of detergent containing no protein (Figure 4.2 D, F), implying that a large exothermic



Scheme 4.1. Representation of the different processes that occurred upon injection of detergent/MPs into APols solutions.

effect is associated with the overall interaction of detergent/MP with amphipols. The magnitude of this effect can be established by subtracting the contribution due to the dilution of free detergent into polymer solution from the signal measured upon dilution of detergent/MP complexes into polymer. The heat associated with the trapping of MP into polymers is presented in table 4.3 and calculated using the equation below:

Q (trapping) = Q (measured in the presence of protein) – Q (measured in the absence of proteins)

Knowing the heat of trapping, one can subtract the heat of formation of mixed micelles between the amphipols and detergents and the detergents released from the IMP/detergent complexes to obtain $Q_{exchange}$, given in equation 3:

$$Q_{exchange} = Q_{trapping} - Q_{mixed} (N_{tot} - N_{buffer}) \qquad \textbf{(3)}$$

where N_{buffer} is the number of mol detergent added to the buffer, and N_{tot} is the number of total mol detergent added including the detergent initially bound to the protein.

Surprisingly, the heat of trapping is nearly identical for a given protein and protein concentration, independently of the structure and concentration of the polymer (1 g/l or 5 g/l) (Table 4.3). This suggests that an excess of amphipol is present throughout the titration and that complete conversion to polymer/MP complexes was achieved.

Table 4.3: Molar enthalpy of MP trapping with amphipols.

MP (**g L^{-1}**)	**APols** (**g L^{-1}**)	**Q trapping** (**kJ mol^{-1}**)	**Q exchange** (**kJ mol^{-1}**)
OmpA (1.2 g L^{-1})	C22-43 (1.0 g L^{-1})	-0.18*	-0.25*±0.05
(1.2 g L^{-1})	C22-43 (5.0 g L^{-1})	-0.16*	-0.48*±0.10
(1.2 or 0.6 g L^{-1})	A8-35 (1.0 g L^{-1})	-0.36 or -0.18	-0.49±0.08 -0.31±0.05
(1.2 g L^{-1})	A8-35 (5.0 g L^{-1})	-0.22	-0.71±0.09
BR (0.9 g L^{-1})	C22-43 (1.0 g L^{-1})	-1.59*	-1.63*±0.1
(0.9 g L^{-1})	C22-43 (5.0 g L^{-1})	-1.88*(-2.52)	-2.03* (-2.66)±0.12
(1.0 g L^{-1})	A8-35 (1.0 g L^{-1})	-1.25	-1.31±0.10
(1.0 g L^{-1})	A8-35 (5.0 g L^{-1})	-0.92	-1.14±0.12

* Within uncertainty, the same Qech was obtained with protein samples diluted by a factor of 2.

A comparison of the data obtained (from figure 4.2, table 4.2 and table 4.3) leads to several observations: first, the replacement of the detergent with amphipols is an exothermic process, suggesting liberation of heat upon binding of the amphipols to MP. Second, the dilution of the complexes detergents/MPs comprises a contribution that corresponds to the detergent dissociation from MP, which can be compared to the detergent demicellization. Third, it provides a strong indication of the similarity of the properties of A8-35 and C22-43 in terms of complexation energetics. Fourth, the exchange heat is different from the heat of demicellization (-2926 to -5016 J/mol for the exchange heat of C8E4 on the surface of OmpA (ΔHdemicellization= -16720 J/mol), and -16720 to -25080 J/mol OTG to BR (ΔHdemicellization= -5434 J/mol). The dissimilarity between the demicellization heat and the exchange heat is an indication that the protein environment changes as result of the interaction between protein and detergent, the association of the protein with amphipols, or a modification of the protein structure. Also, the presence of the protein may alter the number of detergent molecules in the polymer-detergent micelle, yielding a contribution to $Q_{trapping}$ and $Q_{exchange}$.

4.5.2. Dissociation of APols/MP complexes and re-trapping into detergent

Effect of diluting FApol/tOmpA or C22-43/tOmpA solutions with surfactants free buffer. The enthalpies of micellization and critical micellar concentration of detergents and amphipols in absence of protein were studied in order to compare the energy involved in the assemblies in presence or absence of the proteins.

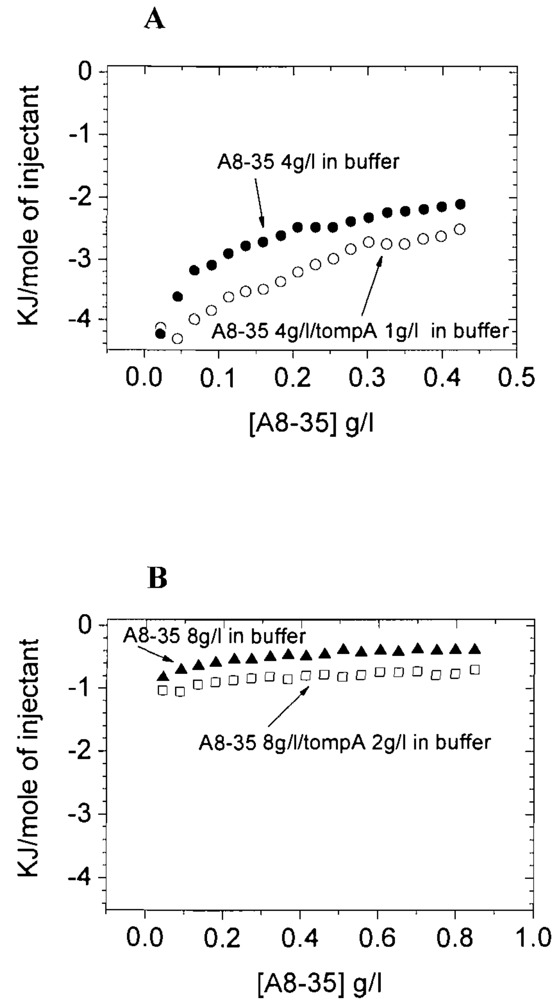


Figure 4.3: Dilution of Apols and APols/tOmpA solutions with surfactants free buffer. (A) Injection of A8-35/tOmpA (tOmpA 1 g L^{-1}, A8-35 4 g L^{-1}) into Tris buffer (○), and corresponding injection of A8-35 (4 g L^{-1}) into Tris buffer (●). (B) Injection of A8-35/tOmpA (tOmpA 2 g L^{-1}, A8-35, 8 g L^{-1}) into Tris buffer (□), and corresponding injection of A8-35 (8 g L^{-1}) into Tris buffer (▲).

Following the injection of FApol/tOmpA or C22-43/tOmpA complexes in surfactant-free buffer, the heat released is very small and equal to the heat of dilution the amphipols into buffer (Figure 4.3), denoting a weak exothermic process (between 0.418-0.836 J/g of amphipol after subtracting the dilution of amphipol solution of the same concentration into the same buffer). The heat evolved remained constant over a wide dilution range (the first injection gives a final polymer concentration of 0.04 g/l) (Figure 4.3). Thus, extensive dilution (140 times) of the amphipol/MP complexes does not induce any detectable desorption of the amphipols from the protein surface, at least in the range of concentration tested. This observation strongly suggests that the binding of amphipols (A8-35 and C22-43) to the transmembrane region of tOmpA, is irreversible under our experimental conditions, at least after 140 times dilution in a surfactant-free buffer.

Exchange of protein-bound amphipol for surfactant. The exchange and desorption of amphipols from a purified complexes amphipols/tOmpA, by addition of a competitive detergent (C8E4) was studied next. In a first set of experiments, a solution of C8E4 (100 mM, higher than the cmc of the detergent) was added gradually (2 μl per injection) to solutions of APol/tOmpA complexes of different concentrations (1.4 ml). The same detergent solution was added under the same conditions to amphipols solution in the absence of tOmpA. The heat evolved was approximately the same in both cases (Figure 4.4).

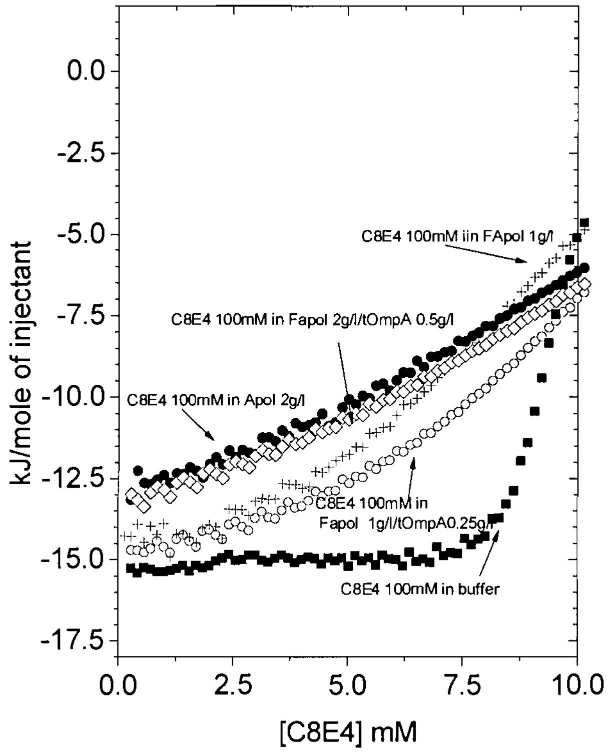
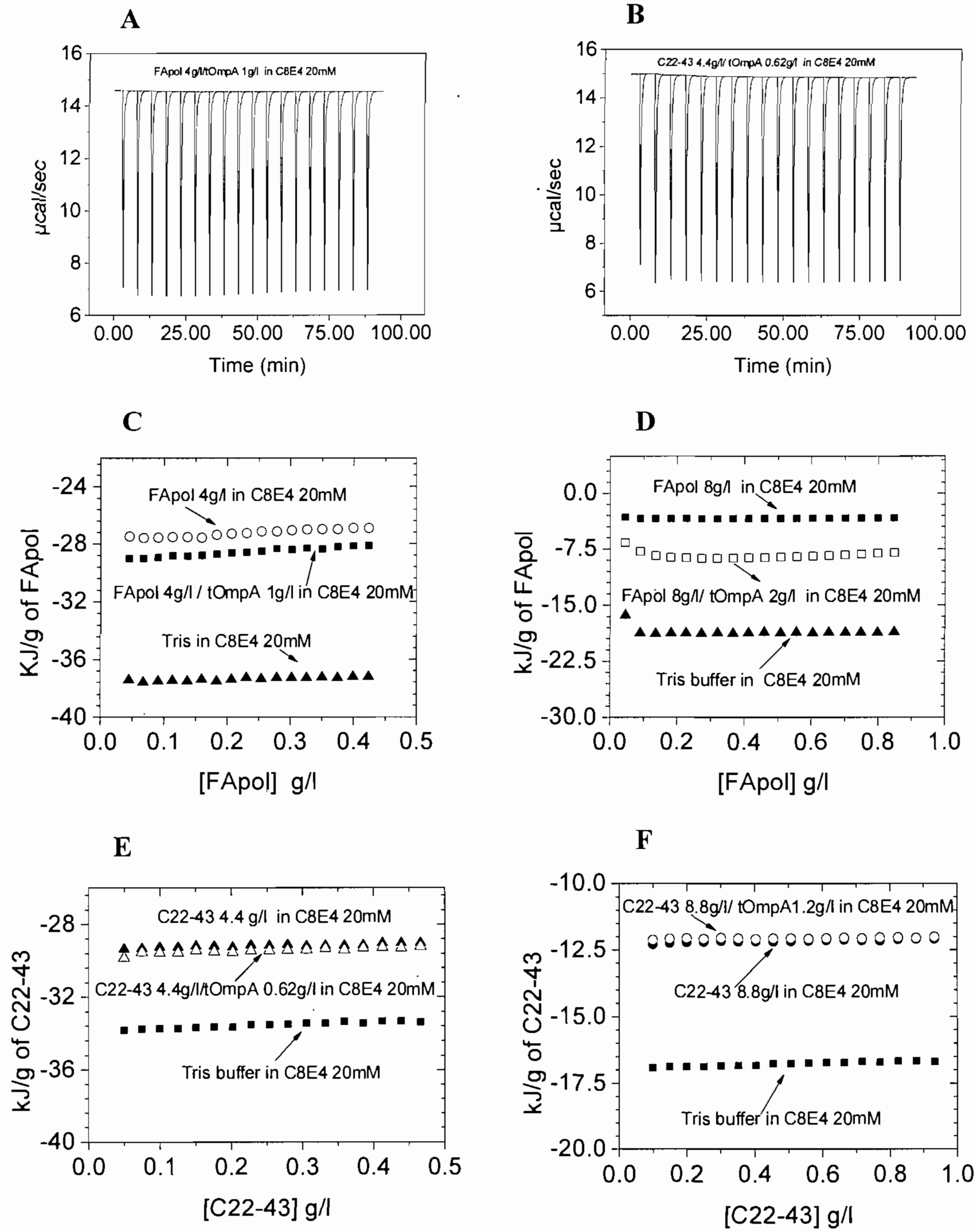


Figure 4.4: Enthalpy of exchange between amphipols and C8E4 on the surface of OmpA. Injection of C8E4 100mM into Tris Buffer (■), into FApol 1 g L^{-1} (+), into FApol 2 g L^{-1} (●), and corresponding injections into FApol/tOmpA (tOmpA 0.25 g L^{-1} and FApol 1 g L^{-1}) (○) and into FApol/tOmpA (tOmpA 0.5 g L^{-1} and FApol 2 g L^{-1}) (◇).

This point is of high importance because it suggests that the detergent (C8E4) places between the water and the amphipols, and the complexes amphipols/IMP show no particular affinity to the detergent in the presence of amphipols. The desorption of the amphipols from the protein surface does not occur in the presence of C8E4 below or above its cmc (Figure 4.4).

We have also conducted a set of experiments to test the ability of the detergent to displace amphipols molecules from the surface of MPs. This was done by injecting different concentration of APol/MP complexes into large excess of detergent solution (above its cmc, the dilution factor varied from 1:140 to 1:17, which corresponds to a micellar detergent/protein ratio of 500 to 60 g/g) (Figure 4.5). Amphipols solutions (without tOmpA, and at the same concentration of amphipol in complexes solutions) were also injected into the same surfactant solution. As shown in figure 4.5, the heat evolved (amphipols alone and amphipols/tOmpA) is independent of the injections number (Figure 4.5 A, B), but depends on the concentration of the MP in the complexes (Figure 4.5 C, D, E, F) or the concentration in polymer (Figure 4.5 C, D, E, F). The heats measured in the presence of the protein and its absence were identical within the experimental error (Figure 4.5 C, D, E, F, and Table 4.4). This indicates that there is no measurable heat due to either the release of amphipols or the binding of the detergent to the protein.



Caption on page 238

Figure 4.5: Enthalpy of exchange of protein-bound amphipol for C8E4. (A) Raw data for the titration of FApol/tOmpA (tOmpA 1 g L^{-1}, FApol 4 g L^{-1}) into C8E4 (20 mM), and (B) for the titration of C22-43/tOmpA (tOmpA 4.4 g L^{-1}, C22-43 4.4 g L^{-1}) into C8E4 (20 mM). (C) Injection of FApol/tOmpA complexes (tOmpA 1 g L^{-1}, FApol 4 g L^{-1}) into C8E4 (20 mM) (■), and corresponding injection of FApol (4 g L^{-1}) into C8E4 (20 mM) (○), and of Tris buffer into C8E4 (20 mM) (▲). (D) Injection of FApol/tOmpA complexes (tOmpA 2 g L^{-1}, FApol 8 g L^{-1}) into C8E4 (20 mM) (□), and corresponding injection of FApol (8 g L^{-1}) into C8E4 (20 mM) (■), and of Tris buffer into C8E4 (20 mM) (▲). (E) Injection of C22-43/tOmpA complexes (tOmpA 0.62 g L^{-1}, C22-43 4.4 g L^{-1}) into C8E4 (20 mM) (Δ), and corresponding injection of C22-43 (4.4 g L^{-1}) into C8E4 (20 mM) (▲), and of Tris buffer into C8E4 (20 mM) (■). (F) Injection of C22-43/tOmpA complexes (tOmpA 1.2 g L^{-1}, C22-43 8.8 g L^{-1}) into C8E4 (20 mM) (●), and corresponding injection of C22-43 (8.8 g L^{-1}) into C8E4 (20 mM) (○), and of Tris buffer into C8E4 (20 mM) (■). In order to facilitate the comparison of the enthalpies in the presence and absence of tOmpA, the *x*-axis refers to the concentration of APols in the samples injected. Temperature: 25°C.

Table 4.4: Enthalpies of dilution of amphipols micelles and complexes of APols/tOmpA in solution of C8E4 (20 mM).

APols	Q_{Apols} **J/g**	$Q_{\mathrm{Apols/IMP}}$ **J/g**
C22-43 (4.4 g L^{-1})	5.85	5.85
C22-43 (8.8 g L^{-1})	4.84	4.85
FApol (4.0 g L^{-1})	10.7	11.2
FApol (8.0 g L^{-1})	7.03	8.03

4.6. Discussion

We have examined the strength of the association of amphipols/MP and the origin of amphipol and detergent attachment on proteins. First, we have compared PC-amphipols with A8-35 at the stage of trapping, which involves the exchange of detergent for amphipol. Second, we have evaluated to which extent the binding of amphipols (A8-35 or C22-43) to the protein can be considered irreversible in a surfactant-free buffer, and if the protein bound amphipol can be exchanged with detergent. The analysis of the exchanges and competitions involved in mixtures of amphipol, detergents and MP was conducted using the regular mixing theory (Bromberg *et al.*, 2000; Couderc *et al.*, 2001; Diab *et al.*, 2007) and by interpreting the heat evolved from these experiments.

Trapping of MP bound detergent by amphipols. Trapping of MP in amphipols necessitates the detachement of the detergent molecules below the CMC, and resembles somehow a demicellization process. In this case, the heat of desorption of the detergent initially attached to the protein and the heat of exchange of detergent to amphipol are measured. Assuming that the exchange of MP to amphipols does not evolve measurable heat, the values obtained for the enthalpy of detergent desorption should be similar to the demicellization enthalpy; but they are not. They were much less for C8E4/tOmpA (~ -5016 J/mol instead of ~ -16720 J/mol), and much higher for OTG/BR (~ -16720 J/mol instead of ~ -5434 J/mol). The difference may be attributed to fast desorption of the detergent from the protein surface and a slow adsorption of amphipol to the protein surface to form a dense layer. After surfactant desorption, the driving force of the interactions is the removal of hydrophobic residues of the MPs from the unfavourable environment of the aqueous phase by binding to the polymer and the displacement of water structured molecule from the close vicinity of the surface.

Diluting APol/tOmpA solutions in surfactants free buffer. This was done to test if in the absence of a competing detergent, the association of APols with MPs is irreversible upon dilution. The injection of APols/tOmpA complexes solutions into buffer free surfactants showed no evidence for desorption of MP-bound APol when the concentration of free APol was lowered (Figure 4.3), by as much 140 times (first injection). The heat evolved was almost similar as that corresponding to the injection of amphipol into protein-free buffer (Figure 4.3). This similarity suggests that dilution of the complexes does not induce any change in the complexes structure. However, aggregation could occur upon dilution because of lowering free APol concentration. The aggregation phenomenon is usually associated with heat and increased exothermicity (Cooper & McAuley-Hecht, 1993). Since increasing gradually the concentration of the protein in the sample cell (by injecting more) did not change significantly the heat evolved, we concluded that complexes are unaffected and no aggregation occurs upon dilution (Figure 4.3). Complexation seems irreversible by addition of amphipols and diluting in surfactant-free buffer, which is further indication of the strength of binding via multipoint attachement of the APols to the hydrophobic surface of MPs. Another factor that may contribute to the irreversibility is the occurrence of structural changes of the proteins upon adsorption. Hydrophobic protein folding, which opposes the conformational freedom of the protein chain, is favourable as long as proteins are in aqueous solutions, while the parts of the protein that are in the vicinity of the surface can bind to the hydrophobic region of the polymers.

Exchange of protein-bound amphipol for surfactant. The dilution experiments have shown that it is not possible to desorb Apols/MPs complexes by simple dilution. Dilution in surfactants solutions, which places the APol/MP complexes in the presence of molecules that can compete with APols and displace them from the surface of MPs, a processus known as un-trapping of amphipols (formation of detergent/MP complexes from amphipols/MP ones), was evaluated. The process was found to be quasi-athermal and the heat generated upon injection of APols/MP complexes into surfactant solutions was almost identical to that of the injection of APols alone. The athermal nature of this process is an indication that the amphipol does not demicellize upon exchange of amphipols for detergent in the un-trapping process. APols have a low critical association concentration (CAC) (<0.04 g/l for C22-43, and < 0.1 for A8-35) and exist always as assemblies of a few polymers chains, even after the first injection (dilution 140 times). Therefore, in the presence of detergent, mixed micelles may forms via interaction of the detergent placed in the sample cell above its cmc, a process, studied earlier and found to be also athermal (Diab *et al.*, 2007).

Advantages of amphipols vs detergents. All results converge to a description of the complex formation having characteristics of a micellization process. Thus, dilution below the CAC of amphipols should induce its desorption from the protein surface (demicellization). This does not occur, implying that the polymers are attached strongly to the protein surface, and that the APol-trapped MPs can be extensively diluted into surfactant-free aqueous buffer without losing APol protective suit. This inherent property offers an excellent stability to the complexes APol/MP without APols dissociation from the surface of MPs. The stability of the complexes toward dilution has been studied recently by Förster resonance energy transfer (FRET) (Zoonens *et al.*, 2007). This is also consistent with studies done by Hong and Lakey, in which OmpF/A8-35 complexes adsorbed onto a solid support were exposed to a flux of surfactant-free buffer after the interaction between OmpF immobilized on the surface and the protein Colicin; this study cannot be done in the presence of detergent (Hong *et al.*, 2001). The stability of the complexes upon dilution makes it possible to use immobilized APol-trapped MPs for investigating MP interactions with biological macromolecules or drugs in surfactant-free buffers.

4.7. Conclusion

This study was aimed at a determination of the thermodynamics underlying the interaction of two types of MPs with two APols and two detergents. Isothermal titration calorimetry (ITC) provided entry into the heats evolved upon exchange of detergent to amphipol, and respectively amphipol to detergent. The energy of the conversion of detergent/MP complexes into APol/MP complexes is of the order of magnitude of the energy of demicellization, whereas conversion of APol/MP complexes into detergent/MP complexes is quasi-athermal. The APol/MP complexes are stable even after dilution 140 times. This clearly demonstrated that APol molecules bind the protein surface in a quasi-irreversible manner. The heat associated with the exchange and dilution phenomena are similar and do not depend on the structure of APols, emphasizing the importance of the hydrophobic interaction in the mechanism of complexation of MPs by APols over interactions of hydrophilic groups of the polymer with water molecules. This is consistent with the fact that the polymers interact preferentially with the MPs surface (Zoonens *et al.*, 2005).

This study is important to understand the stability upon dilution of the Apols/MPs and the thermodynamic phenomenons involved when preparing APol/MP complexes by competitive exchange with protein-bound detergent. It provides a compilation data that we expect to be useful for experimental and theoretical studies of membrane protein intended to improve the understanding of MPs structures and functions. It establishes that replacing detergents by APols is a viable approach to the study of MPs, since it prevents their precipitation and inactivation.

4.8. Acknowledgements

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4.9. References

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CHAPTER FIVE

GENERAL DISCUSSION

5.1. Solubilization approach (Figure 5.1)

Solubilization is defined here as the formation of a thermodynamically stable solution of a solute that is normally insoluble or very slightly insoluble in water via the introduction of one or more amphiphilic component(s) (Attwood *et al.*, 1983). Solubilization, using surfactants and amphiphilic polymers, has played a vital role in pharmaceutical research especially in the development of many pharmaceutical formulations with highly hydrophobic drugs (Malik *et al.*, 1975; Nerurkar *et al.*, 1996; Patist *et al.*, 1998; Zana, 2002). In the present work, amphiphilic molecules (surfactants and polymers) have been used to manipulate integral membrane proteins (IMPs), which are very hydrophobic and insoluble in aqueous solution. Solubilization of IMPs is a process in which the proteins and lipids that are held together in the biological membranes are dissociated in a detergent solution (Figure 5.1) (le Maire *et al.*, 2000). An essential criterion for the solubilization of IMPs is that the proteins must be carefully removed from the native membrane to preserve them in their functionally active form; this ability will represent an important step for understanding the structure and the function of IMPs for their biomedical application. However, IMPs tend to denature and aggregate in the presence of commonly used detergents (Jones *et al.*, 1999). This remains a major difficulty for the handling of proteins for biochemical assays and structure analysis. This problem has prompted the design of polymeric surfactant, called amphipols (Tribet *et al.*, 1996; Popot *et al.*, 2003), which do not extract IMPs directly from biological membranes but can replace detergents after solubilization (Figure 5.1).

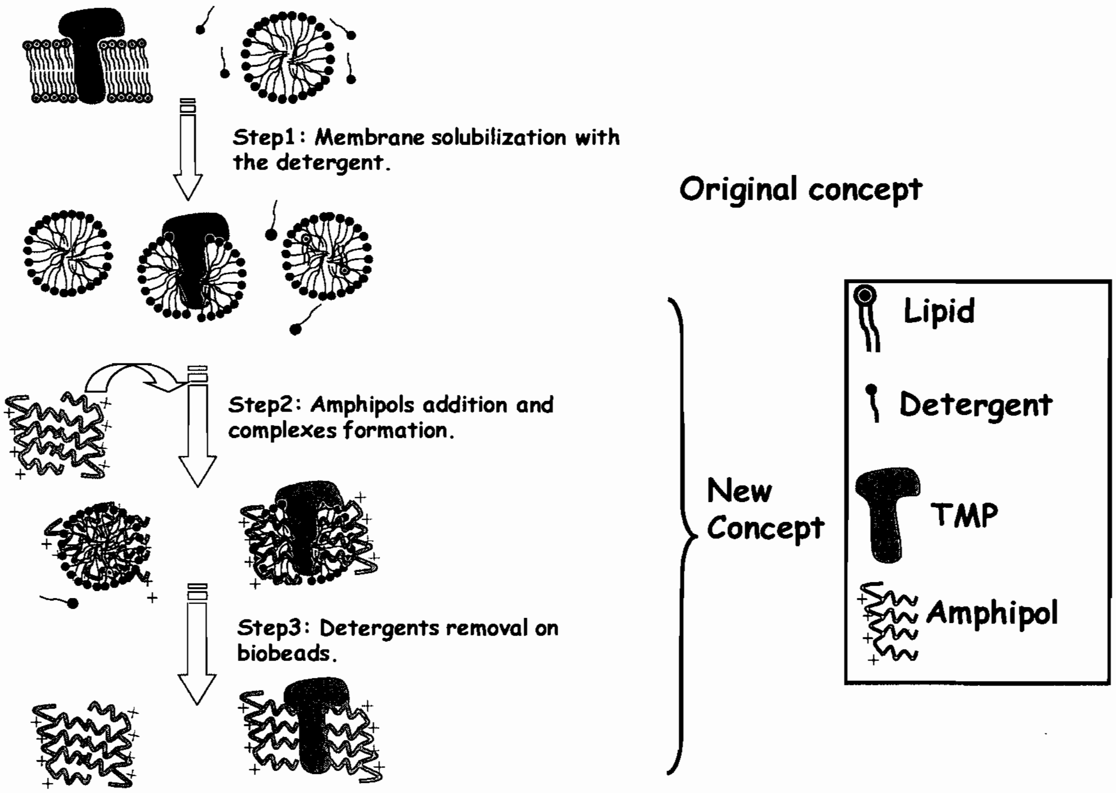

Figure 5.1: Solubilization approach.

Amphipols bind with the transmembrane domain of IMPs with multiple attachement points, presumably in a way such that the hydrophobic groups of the polymers interact with the hydrophobic surface of the protein while the polar groups provide a hydrophilic corona and thereby maintain the colloid stability of amphipol/IMP complex and expand the range of conditions in which IMPs can be manipulated.

5.2. Polymers

5.2.1. Polyacrylate-based polymer (A8-35)

Polyacrylate-based polymers amphipols are amphiphilic molecules designed to replace detergent in integral membrane protein solution studies (Tribet *et al.*, 1996). They are random copolymers of *n*-octylacrylamide, *N*-isopropylacrylamide, and acrylic acid (A8-35). In this study, the A8-35 amphipol (A stands for anionic, "8" refers to the average molar mass (in kg mol^{-1}), and '35' is the molar percent of carboxylate) was employed (Figure 5.2 A), because it has been extensively studied. Due to its high hydrophobic content, A8-35 moleules in water organize into well-defined, small, near spherical micelle-like particles, with a Stokes radius of ~3 nm and a molar mass of 40 kDa (Figure 5.2 B). Detailed studies of the solution properties of A8-35 are available (Gohon *et al.*, 2006). The data presented are of particular interest and suggest that A8-35 can provide a dense, packed layer that can protect the highly hydrophobic transmembrane domain from aqueous solution. However, the use of A8-35 has two drawbacks. First, the polymer has a net charge, which prevents the use of IMP/A8-35 in some experimental situations, such as the ion-exchange chromatography and high Ca^{2+} content (Picard *et al.*, 2006).

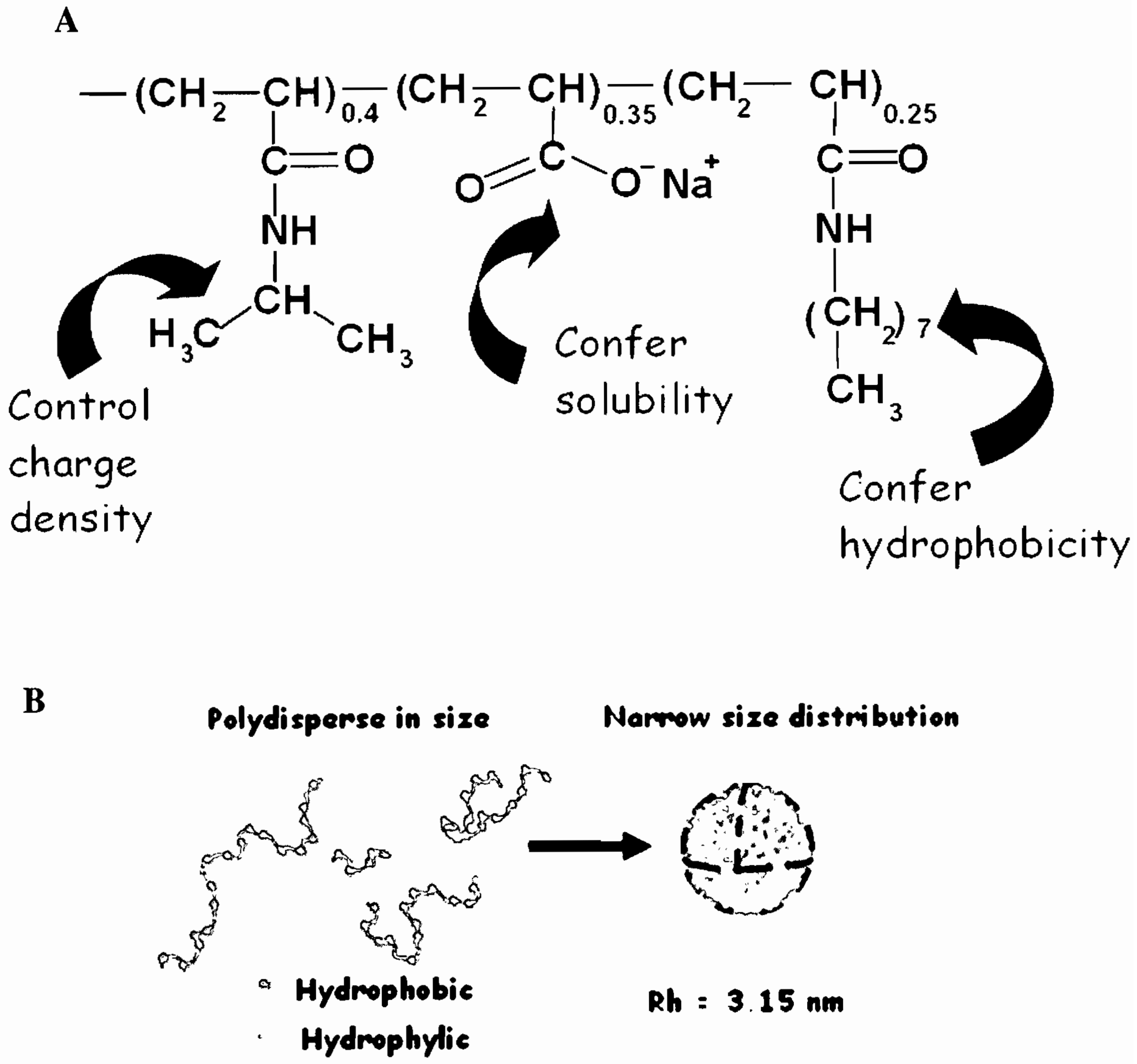

Figure 5.2: (A) Chemical structure of A8-35. (B) Representataion of A8-35 assemblies in water.

This constraint is likely to be a burden in crystallization where excess of salts is needed. Second, A8-35 is a weak acid, which confines its use to solutions of neutral or basic pH, since protonation of the carboxylates under acidic pH condition, induces polymer aggregation and precipitation. This observation emphasizes the fact that the physical and biochemical properties of amphipols/IMPs complexes depend on the specific chemical structure of amphipols. Therefore, tailoring the structure of amphipols can be used to alleviate some of the problems encountered with A8-35.

5.2.2. PC-based amphipols (C45-68, C60-30, C60-44, C22-43)

The strategy used in this work to enhance the solubility of amphipols in buffer of high salinity and acidic pH involves the substitution of carboxylates of A8-35 by units that possess covalently linked cationic and anionic groups (phosphorylcholine). The four PC-based polymers prepared (C45-68, C60-30, C60-44, C22-43) are random copolymers of n-octylacrylamide, *N*-isopropylacrylamide, and *N*-phosphorylcholine-*N'*-ethylenedioxybis(ethyl)acrylamide (Figure 5.3 A). They are cationic in solutions of pH below 9 as a consequence of the presence of a secondary amine in the fragment linking the PC group to the polymer chain (Figure 5.3 B). The positive charge on the polymer is believed to have no effect on either the polymer properties in solution or on the polymer/IMPs interactions, due to its location close to a bulky group, phosphorylcholine group that provides a shield-like environment (Figure 5.3B), which protects this positive charge from any electrostatic interactions with other groups or molecules.

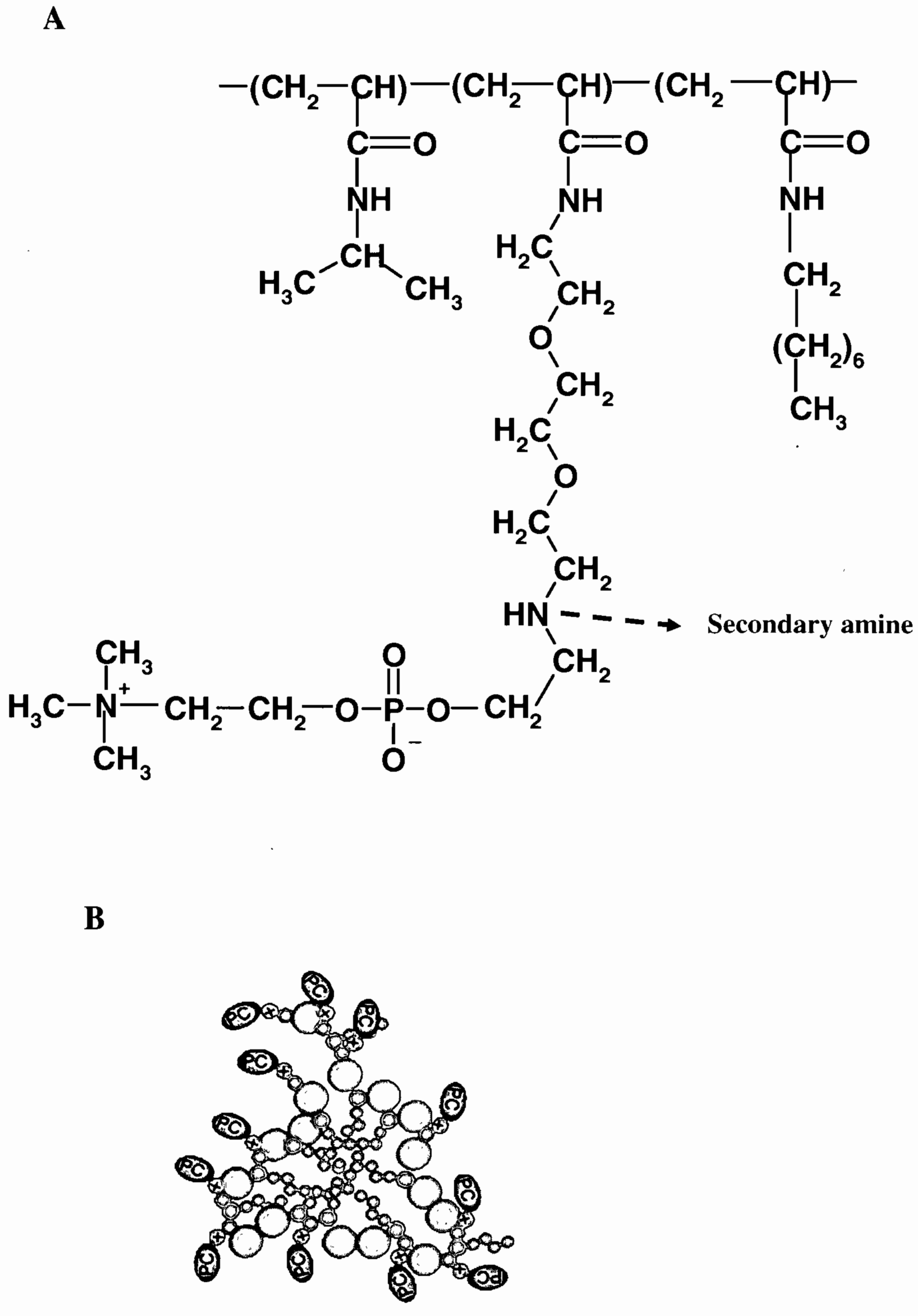

Figure 5.3: (A) Chemical structure of PC-based polymers. (B) Representataion of PC-based polymers assemblies in water.

PC-based polymers self-associate in water (Figure 5.3 B). The size of the assemblies is related to the polymer structure but independent of polymer concentration at least within the experimentally accessible range 1.0 to 20.0 g/L. The polymers C60-44, C60-30, and C45-68 (having no *N*-isopropylacrylamide units along in its backbone), which were synthesized by standard free radical polymerization, have a larger molar mass and their molecular distribution is broader, as compared to C22-43, which was prepared by controlled free radical polymerization. This difference in the structure of the polymers induces a significant difference in the size of their assemblies in aqueous solutions. C22-43 forms species having a Stokes radius of 4.4 nm similar to the size of IMP and of detergent micelles. C60-44 and C60-30 assemble into objects, which have a Stokes radius of ~15 nm and a broader size distribution, as compared to C22-43 particles. A small fraction of large aggregates ~150 nm could be detected in solutions of C60-44 and C60-30. These aggregates can be removed effectively by centrifugation. C45-68 assembles into larger objects, which has a Stokes radius of 24 nm and a broad size distribution; thus C45-68 is not suitable for use in integral membrane protein studies, where small size and narrow micellar size distributions are required.

Despite of the advantages that PC-polymers can provide, there exist some negative aspects. First, the cationic nature of the polymers makes them highly cytotoxic, since the cell surface is anionic and the polymers can interact non-selectively with the cells and adsorb on its surface. This represents a major barrier if the polymers to be used in the delivery of IMP to the membrane or in any in vivo applications. Also, the net charge can prevent the use of the complexes PC-amphipols/IMP in many experimental situations, such as capillary electrophoresis where the capillary is negatively charged and the complexes can adsorb on the capillary surface and complicate the analysis. The net cationic charge on the polymers is due to that the phosphoryline units were attached to the polymer by reductive amination of phosphorylcholine glyceraldehyde following a protocol described previously (Miyazawa & Winnik, 2002). The cationic nature of the polymers could be avoided by linking the phosphorylcholine units to the polymer by an amide bond. This could be done by oxidizing the phosphorylcholine glyceraldehyde into its acid and then link the resulting acid to the polymers in the presence of a coupling agent, such as carbonyldiimidazole. Another drawback, which is specific to the polymers prepared by RAFT polymerization, is that they carry a thiocarbonylthio group at one chain end. This end group is colored and absorbs in the UV at the same wavelength of the aromatic residues of proteins. This tends to add difficulties to the obtained spectra and its interpretation. The removal/transformation of the trithiocarbonylthio end groups can be achieved by one-pot reaction developed recently in Winnik group (Qiu & Winnik, 2006). The procedure involves first the aminolysis of the thiocarbonylthio groups with an amine (butylamine), and second the transformation of the resulting thiols into thioethers by reacting the obtained polymer with α, β-unsaturated ester (2-hydroxyethylacrylate or butyl acrylate). This treatment was done for the polymer C22-43 used in the present study. The trithiocarbonate group was almost completely removed (>99%) confirmed by UV-vis spectroscopy using the characteristic absorbance centered at 310 nm for the trithiocarbonate group (Figure 5.4).

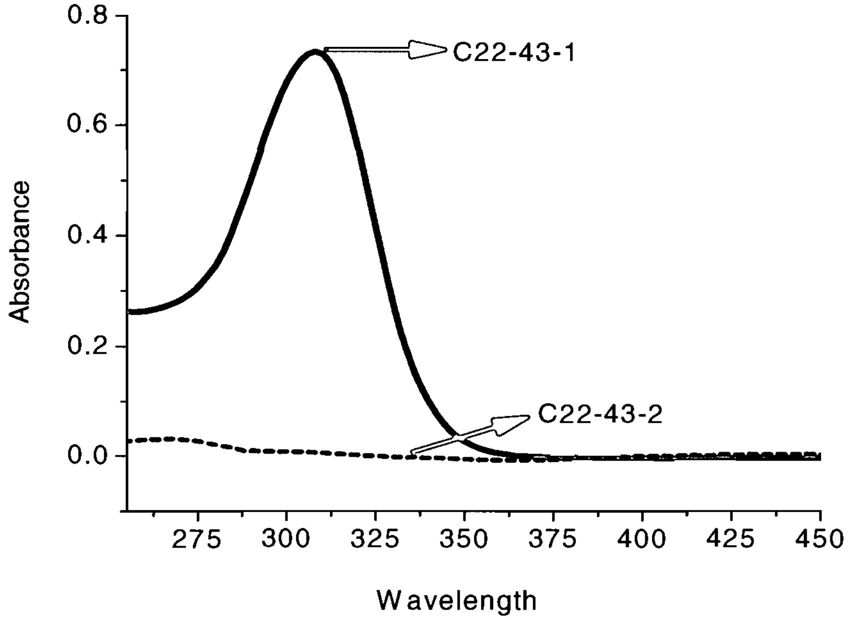

Figure 5.4: UV-vis spectra of the precursor RAFT polymer C22-43-1 (Red, solid line) and the aminolyzed polymer C22-43-2 (Blue, dash line), concentration: 1g L^{-1} in chloroform.

The original polymer exibits a strong absorption at 310nm, while no absorption can be detected for the resulting polymer after aminolysis (Figure 5.4).

5.3. Integral membrane proteins selection

Integral membrane proteins carry out some of the most important functions in living cells, yet few details are known about their structures. This is due, in large part to the difficulties associated with preparing membrane proteins in a way suitable for their analysis. Consequently, much effort has been devoted to overcome the problem of solubilization and stabilization of different types of IMPs. α-Helical integral membrane proteins account for approximately 20% of all proteins encoded in the genome of cells (Wallin & von Heijne, 1998). They are also the therapeutic targets for most of the drugs currently in medicinal use. In the present study, bacteriorhodopsin, a member of this large family, has been used. BR is a proton-transporting IMP in *Halobacterium salinarum*. It is the only protein in the membrane, which facilitates its purification. It is a prototype of membrane transporters and serves as a structure model for the general class of seven-transmembrane G-protein receptors (GPCRs), which are important drug targets. It has been used as a model for investigating the effects of amino acid substitutions on the kinetic and thermodynamic stability of helical membrane proteins. Moreover, BR has a rich and extensive base of spectroscopic and biophysical characterizations of its structure and activity. Its stability can be monitored easily by calculating the ratio A_{280}/A_{554} (corresponding, respectively, to the absorbance of aromatic residues of the BR and the absorbance of native BR, this value should be around 2 for a native BR). Another protein that belongs to the α-helical class, cytochrome *b6f*, has been employed. The cytochrome *b6f* is one of integral membrane protein complexes involved in electron transport in membranes that carry out oxygenic photosynthesis and it is an ideal example of a hetero-oligomeric integral membrane protein whose solubilization, purification and crystallization was not very successful to date.

The third protein used is OmpA, which is a β-barrel transmembrane protein that carries out diverse functions in many organisms. It is found in the outer membrane of bacteria, mitochondria, chloroplasts, and in the transmembrane pores of some secreted protein toxins. Its small size, easy synthesis, and the wealth data available were the main incentive that has driven us to decide on. Among few integral membrane proteins, OmpA has shown to fold from a fully denatured state in detergent solutions (Booth *et al.*, 2001). But, detergents have found to add difficulties to its structural analysis and may interfere with its activity measurement. Replacing the detergent with amphipols has motivated us to use OmpA to improve the structural analysis resolution of β-barrel membrane proteins.

5.4. Solubilization efficiency

In spite of the successful use of detergents for the solubilization of a number of membrane proteins, this approach has some restrictions (le Maire *et al.*, 2000; Bowie, 2001; Garavito & Ferguson-Miller, 2001). Developing a range of alternative structures is of main interest. The ability to obtain desired amounts of solubilized integral membrane proteins will be critical for structural and biochemical analysis of these proteins.

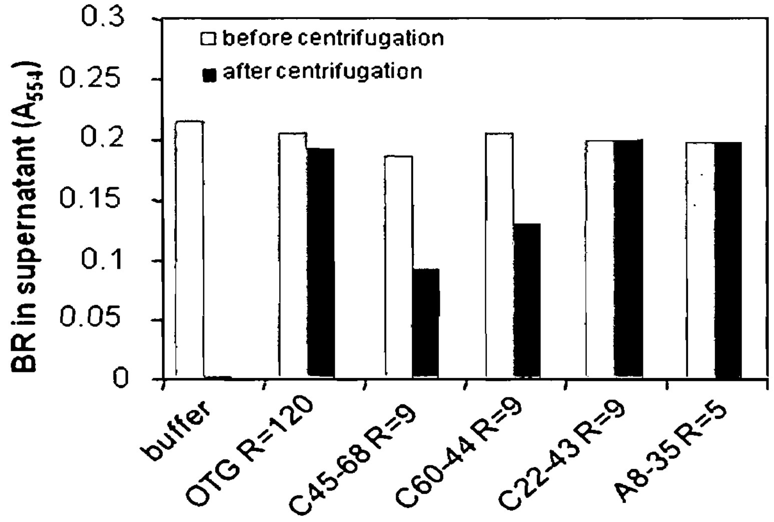

Figure 5.5: Comparison of PC-amphipols at *R* = 9 and A8-35 at R=5. Solubility of BR at submicellar concentrations of OTG in the presence of PC-amphipols or A8-35. Aliquots of a stock solution of polymer were added to the stock solution of BR (~1 g/L BR solubilized in 33 mM OTG, 20 mM phosphate buffer, pH 6.8) to obtain various polymer/BR ratios, *R* (in g/g). After incubation for 15 min at 4°C, samples were diluted 10× in 100 mM NaCl, 20 mM phosphate buffer, pH 6.8, and centrifuged at 200,000 × *g*, 10 min., 8°C, in a Beckmann Airfuge. The fraction of BR kept soluble was determined from the absorbance at 554 nm of the supernatants. "OTG" and "Buffer" refer respectively to dilutions into the phosphate buffer, in the absence of polymer, with and without 33 mM OTG.

Thus, comparison of the structures efficient vs inefficient is crucial and will help to define the design rules of a good amphipol. In the present study, PC-based amphipols, having different composition, molecular weight and polydispersity, were compared at first in terms of efficiency at the stage of trapping of IMPs (Figure 5.5). The ability of the PC-amphipols to solubilize IMPs was also compared with A8-35 the best-characterized amphipols to date. As shown in figure 5.5, in the absence of amphipols, dilution-centrifugation of the OTG/BR complexes results in complete precipitation of the protein. Detergent depletion, without supplementing the solutions with another amphiphile, leaves the protein with no hydrophilic corona that allows the interaction with water to promote solubilization; this in fact induces IMPs precipitation. In contrast, in the presence of either PC-amphipols or A8-35, a significant amount of protein concentration was detected in the supernatant where the polymer/BR ratio was higher than 3g/g. All the protein remained soluble at a ratio of 9 for C22-43 and a ratio of 5 for A8-35 (Figure 5.5). With C45-68, C60-44 and C60-30, a plateau at 70% of protein have reached for polymer/protein weight ratios of 5/1. The decrease in trapping efficiency is believed to be due to the presence of a small amount of large particles which was confirmed using a pre-centrifuged C60-44 solution to prepare the complexes C60-44/BR; this in fact enhanced the solubility of BR to 80%. The fact that BR remained in the supernatant suggest that BR binds to the polymer and forms soluble protein/polymer complexes, thereby preventing its aggregation and precipitation. The mechanism envisaged here is that the hydrophobic part of the polymers interacts with the hydrophobic region of the protein while the hydrophilic part of the polymers interacts with water and ensures the protein solubilization. The amount of protein-bound amphipol, the size and size dispersity of the complexes were found to depend on the hydrophobicity of the polymers and the percentage of phosphorylcholine incorporation. High polymer hydrophibicity provides more attachement point of the polymer to the protein, and covers efficiently the hydrophobic region of IMPs; thus the interaction between the polymer and IMP is stronger, the resulting complexes are more robust and stable and the amount of trapped IMP is higher. In contrast, high phosphorylcholine content (as in C45-68) persuades the formation of intrachain (within single polymer chain) and interchains (between neighboring polymer chain) interactions or ion pairing between phosphorylcholine groups (Figure 5.6). As a consequence, the polymer becomes less soluble and large polymer aggregates form; hence the amount of bound proteins decreases and the size and size dispersity of the polymers/IMP complexes increases. When the complexes were deposited onto a surfactant-free sucrose gradient, a single sedimentation band was observed for the complexes with OTG, C60-44, C60-30, C22-43, and A8-35 (Figure 5.7). The presence of a single band confirms the absence of any aggregation during the entrapment experiment. In the case of C45-68, which has a high phosphorylcholine content, the sedimentation band was much broader and aggregates were observed at the bottom of the gradient (Figure 5.7). The sedimentation band of C60-44/BR and C60-30 is significantly broader than that of OTG/BR, C22-43/BR, and A8-35/BR (Figure 5.7). This is due to the fact that the polydispersity in size of the polymers, which was significantly higher in the case of C60-44 and C60-30.

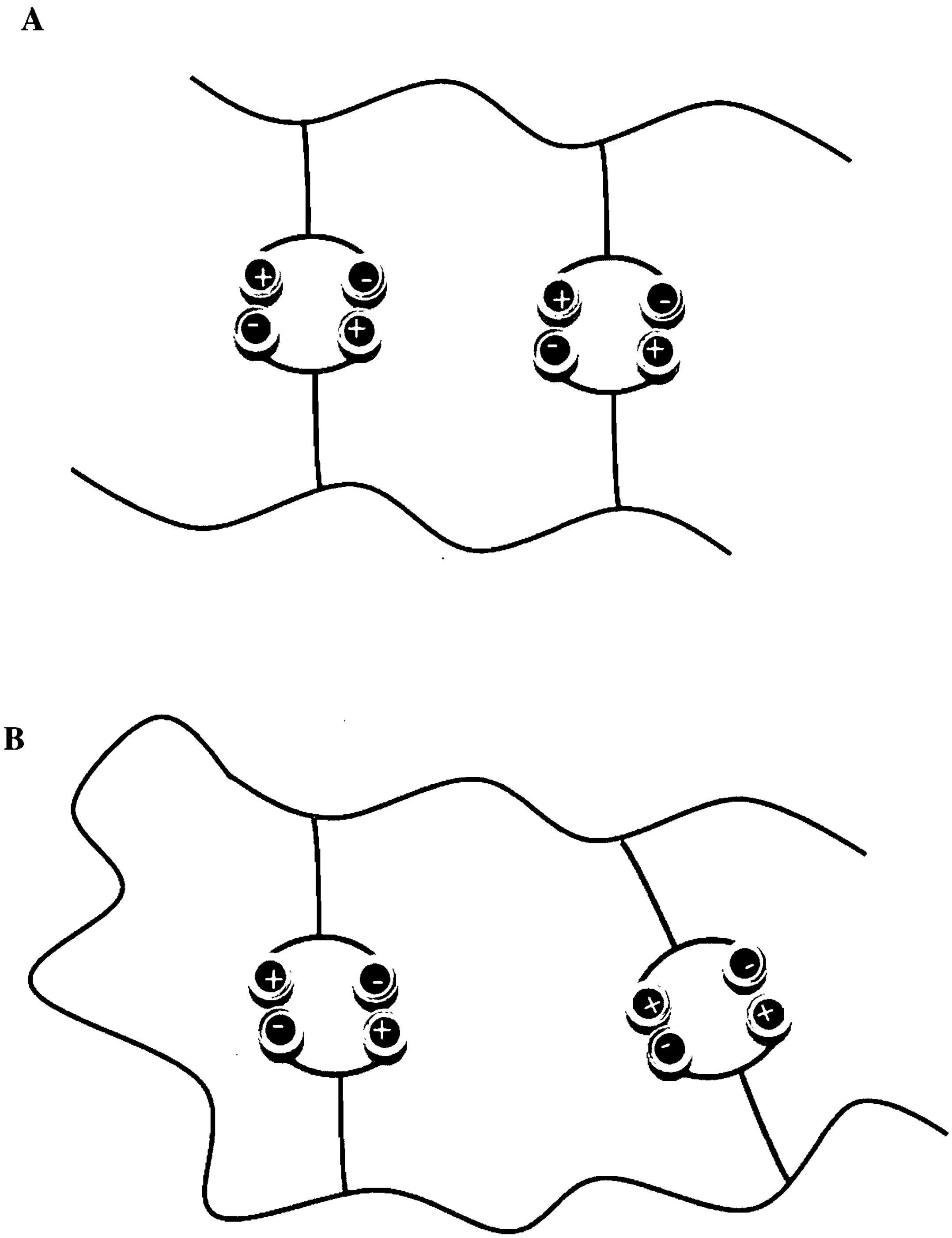

Figure 5.6: Representation of inter- (A) and intrachains (B) interactions in phosphorylcholine-based polymers.

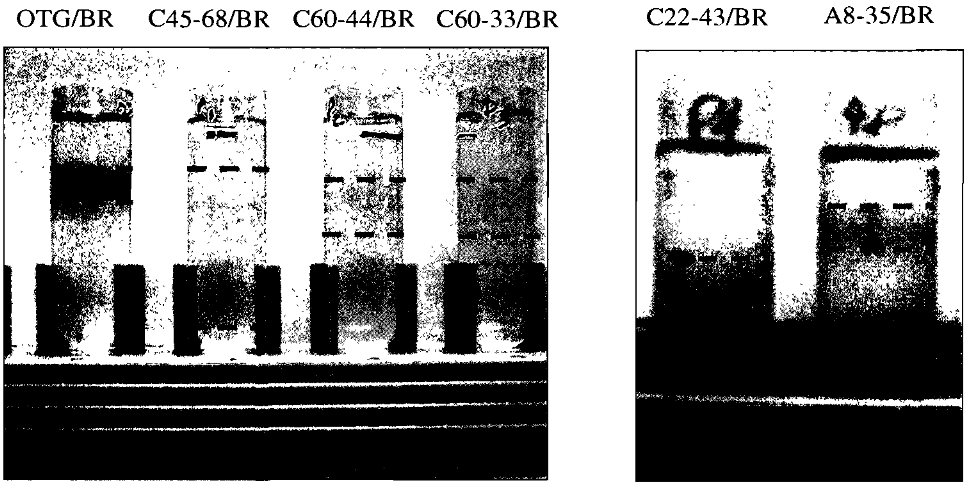

Figure 5.7: Rate zonal sedimentation analysis of complexes OTG/BR, C45-68/BR, C60-44/BR, C60-30/BR, C22-43/BR, and A8-35/BR in 5-20% (w/w) sucrose gradients. Photographs of the tubes after centrifugation. The samples was layered on the top of gradients (20 mM phosphate buffer, pH 6.8), which were centrifuged at 37,000 rpm (Beckman TLA centrifuge, MLS 50 rotor, ~145,000 × *g*).

These observations point out to the importance of the structure, composition and physico-chemical characteristics of amphipols on the characteristics of their complexes.

5.5. Complexes stability

The native structure of IMP is a basic requirement for their functioning. The function of any protein depends on the fact that it can carry on the same structure over an extended period of time in a desired medium. As such, it is imperative to understand what trigger denaturation of a protein and to what extent one can control this through different chemical environment.

The best-characterized amphipol to date, A8-35, has proven its efficiency compared to the detergent (OTG) at stabilizing IMPs over extended period of time (Tribet *et al.,* 1996; Champeil *et al.*, 2000; Picard *et al.*, 2006) (Figure 5.8). A8-35 was not able to maintain IMPs soluble at acidic pH (Figure 5.8), and in the presence of either 1M NaCl or in the presence of calcium ions (Picard *et al.*, 2006; Zoonens *et al.,* 2007). Immediately, all the complexes containing A8-35 became turbid and after 4h, a complete precipitation of the A8-35/BR samples treated with calcium or subjected to low pH conditions was occurred. While in the case of C22-43, C60-44, and C60-30, the samples treated with 1M NaCl or 12mM Ca^{2+}or brought to low pH remained soluble and more than 90% of the BR was maintained native over an extended period of time in all media (Figure 5.8).

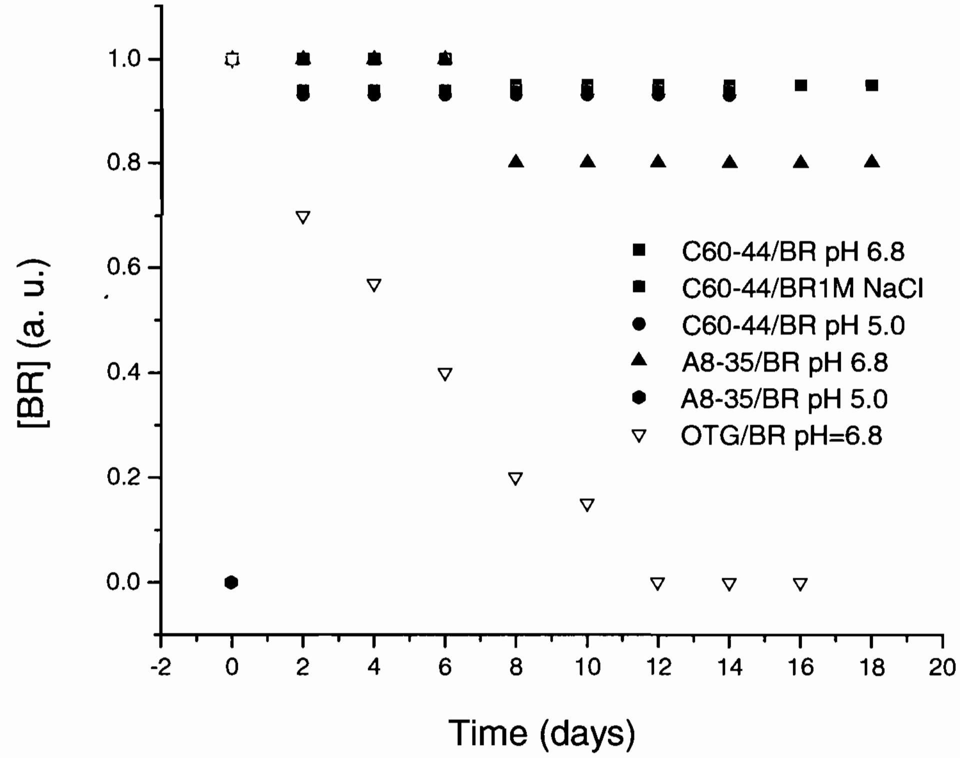

Figure 5.8: Stability of BR complexed with C60-44 (with time, in 1M NaCl, and at pH=5), A8-35 (with time, and at pH=5), and OTG (with time).

The robust nature of the complexes in the case of PC-based amphipols is in fact due to the presence of phosphorylcholine units along the backbone of the polymers. The presence of PC makes them insensitive to the pH change because of the inner salt formation between the phosphate and quaternary ammonium groups in a PC groups. This inner salt structure is stable and is always electrically neutral in aqueous media. Thus, the polymer exhibits a similar behavior in solution at different pH. Their high solubility in saline solutions can be explained in terms of the salt penetrates the ionic networks of the polymers, screens the net electrostatic interactions between the polymer chain (Figure 5.9), increases the repulsion between the polymer chains, triggers chains expansion, and hence promotes the dissolution of the polymer. This behavior suggests strongly the presence of intermolecular association in the absence of salts. This phenomenon can happen for two reasons. First, the inorganic salts (NaCl and $CaCl_2$) stabilize the phosphorylcholine by salt formation. So the number of phosphorylcholine groups, which can interact between them decreases by the addition of salts. The other reason is related to the structure of water in the medium. Na^+ and Ca^{2+} are water structure maker ions, but Cl^- is structure breaking ion which will disturb the water cluster and allow the formation of more hydrogen bonding between the polymer and water and hence increase its solubility (Bockris & Saluja, 1972). The increased solubility in the presence of salts has been observed previously for other polybetaines (Peiffer & Lundberg, 1985; McCormick & Salazar, 1992).

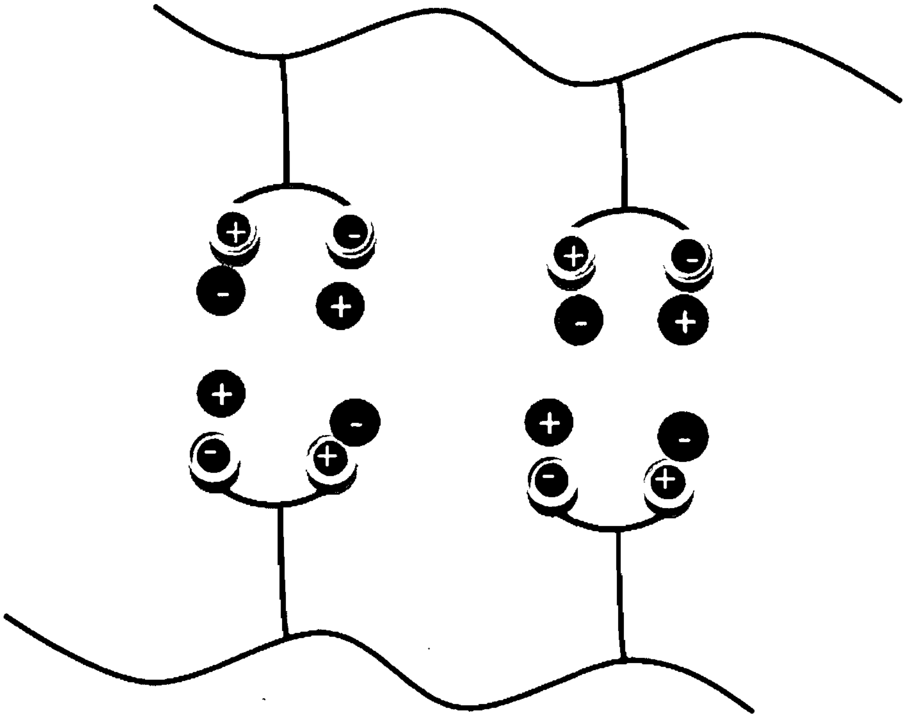

Figure 5.9: Representation of the salts bound to the phosphorylcholine units of the polymer.

Phosphorylcholine polar unit, which is a pH-insensitive group and highly soluble in medium of high salinity and in the presence of Ca^{2+} ions, makes it possible for us to introduce new amphipols that have the capacity to overcome many of the physical-chemical limitations of amphipols available to date.

5.6. Amphipols/surfactants interactions

Interactions between amphiphilic polymers and surfactants have been a subject of increasing interest because of their biological, pharmaceutical, and technological importance (Brackman & Engberts, 1989; Brackman & Engberts, 1991; Goddard & Ananthapadmanabhan, 1992). These interactions are considered to result in the formation of complexes between the surfactants and the polymer due to electrostatic and hydrophobic forces. Studies on polymer-surfactant systems have focused mainly on attractive interactions between oppositely charged polyelectrolytes and surfactants, and between nonionic and anionic polymers and anionic surfactants (Hayakawa & Kwak, 1982; Kwak, 1998; Deo *et al.*, 2003). Little attention has been given to the interaction between neutral surfactants and polymers, since these interactions are very weak and cannot be detected by standard analytical tools (Brackman & Engberts, 1991). Since neutral surfactants are used for the solubilisation of membrane proteins, the main subject of this work, part of this study was aimed to investigate the interaction of these surfactants with amphipols to detect the presence of mixed micelles obtained due to the interaction amphipols-surfactants. The presence of such mixed micelles may affect the stability and the enzymatic activity of amphipols/IMP complexes.

The interactions between amphipols and surfactants are concomitant with a significant change of thermodynamic parameters, due to the occurrence of many processes, involving the overlapping of different types of molecular forces, such as van der Waals forces. The resulting interactions are combinations of such processes and have pronounced effect on the structure of the complexes that are formed. The formation of polymer-surfactant mixed micelles is favoured by an increase in entropy (Blokzijl & Engberts, 1993). This increase could be attributed to the tendency of the hydrophobic groups of the surfactant to remove itself from the solvent environment and aggregate along the hydrophobic group. As water do not form hydrogen bonds with the surfactant hydrocarbon chains, the water molecules form a structure surrounding the hydrophobic groups that results in the formation of cavities in the water structure. The resulting water molecules become more ordered, which in turn attribute to a decrease in entropy. As concentration of the surfactant increases in the solution, aggregation happens when the hydrophobic groups are removed from water. The highly organized water structure returns to normal hydrogen-bonded structure with an increase in entropy that drives the surfactant aggregation process (Némethy & Scheraga, 1962).

According to the light scattering results, the polymers (C22-43 or A8-35) in aqueous solution exists as compact, small, and monodisperse micelle-like assemblies, consisting of two to four polymer chains. Adding the detergent to the polymer solutions, the scattered light intensity increases slightly, claiming the interaction between the polymer and the surfactants and the formation of amphipol/surfactant complexes. The light scattered enhacement is due in fact to the detergent micellization, detergent binding to the polymer, and variation of the average number of polymer chains involved in self-assemblies or in mixed micelles. The molar mass of these assemblies increases weakly below the cmc of the detergent, but a sharp increase were obtained when approaching the cmc of the detergent. This is due to an increase in the availability of hydrophobes for the binding to the polymer, pointing out to a cooperative nature in the binding. In the case of cooperative binding of a small molecule to a polymer having a number of binding sites, the binding of the small molecule at one site increases the affinity of the molecule at adjacent sites. The driving force for the binding here is hydrophobic interactions between hydrophobic parts of the polymer and surfactants. Our results show that the mixed micelles formed, except in the case of OTG/A8-35, remained small (< 5 nm) independent of detergent concentration and they did not form assemblies that are polydisperse in size. Small and monodisperse amphipol micelles are critical in the preparation of amphipols/IMP complexes and have to be controlled during all steps of IMP purification.

Data obtained from ITC gave us access to the thermodynamic parameters associated with binding process, which was analyzed using the binding isotherms obtained by CE. The heat evolved upon injection of a micellar surfactant solution is the result not only for the heat of demicellization but also the heat of interaction between the surfactant and the polymer (Figure 5.10). The enthalpograms obtained with ITC showed that the heat evolved upon injecting the surfactants into polymer solutions was less than in the absence of polymers pointing out to an endothermic contribution.

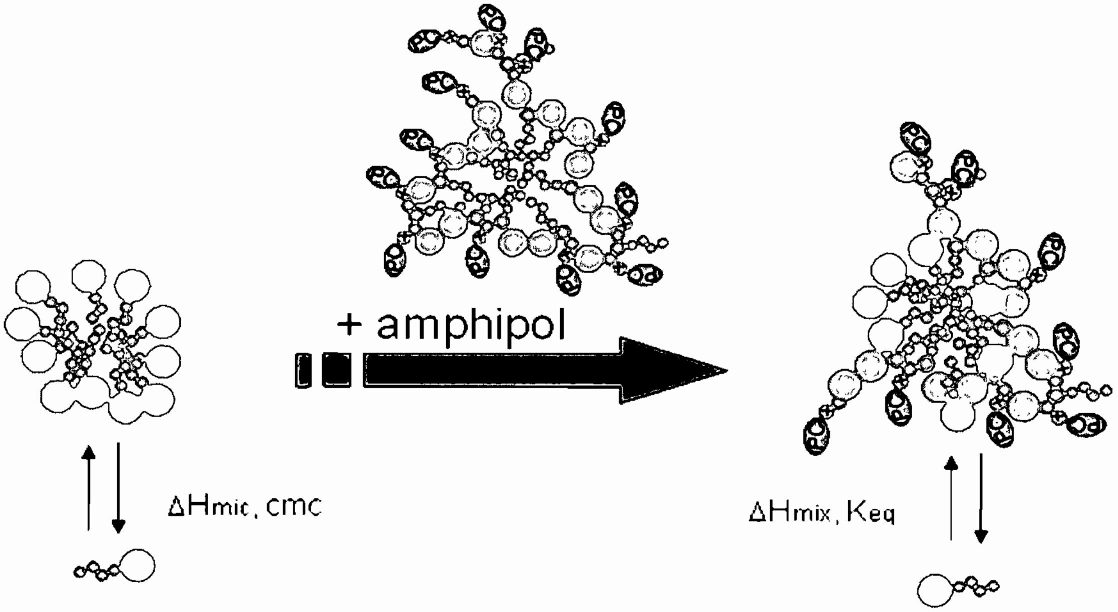

Figure 5.10: Representation of the formation of mixed micelles of PC-amphipols and detergent.

This endothermic effect results from the hydrophobic interactions between surfactant tails and polymer hydrophobic groups. This interaction increases upon increasing the polymer concentration, indicating that the interaction occurs in proportion to the amount of polymer chains in solution. To account for such binding coperativity and to estimate the composition of mixed micelles, two common models were applied in this study, the hill model (Hill, 1910) and the regular mixing model, which are appropriate for multisites binding with cooperativity. Below the cmc of the detergent, the molar fraction of detergent is very low, and the micelles are essentially composed of amphipols. The detergent fraction in mixed micelles starts to increase when the concentration of free detergent approaches the cmc of the pure detergent. This point is of prime importance when preparing polymer/IMP complexes that needs to be depleted of excess detergent because of IMPs' activity is very sensitive to the presence of trace amounts of detergent. To achieve a negligible contribution of the detergent to the amphipols, the concentration of the total detergent must be significantly below the cmc (< cmc/100).

5.7. Exchange between surfactants and amphipols on the surface of MP

The structural characterization and the eventual biomedical of applications of integral membrane proteins makes their solubilization and isolation from the membrane a major stumbling block and a challenging task in current membrane biology. A variety of detergents and polymers have been employed successfully to extract membrane proteins from biological membranes and to keep them soluble in water. The problem encountered with low molar mass detergents is their dissociating character and the obligatory presence of excess micelles in detergent solutions. This indeed complicates the achievement of a high stability. The shortcomings from the detergent can be alleviated through the use of amphipols, since multiple non-polar groups of single amphipol molecule limit in practice spontaneous desorption and make their noncovalent association with the protein quasi-irreversible. The detergency of amphipols is extremely weak and, in general they do not efficiently extract IMPs from biological membranes (Tribet *et al.*, 1996; Popot *et al.*, 2003). However, IMPs solubilized with hydrogenated detergents can be transferred to amphipols by competitive of protein-bound detergent for the amphipols. Little is known on the exchanges and competitions involved in this mixtures of amphipols, detergents and IMPs. This prompted us to investigate the energy of formation and exchange of amphiphile in complexes between various detergents, amphipols and IMPs by isothermal titration calorimetry (ITC). In one hand, the study indicated that the energy of the conversion of detergent/IMPs to APol/IMPs is of the order of magnitude of the energy of demicellization, implying a fast desorption of the surfactant and a slow adsorption of the amphipols to the protein. On the other hand, de-trapping of IMP from amphipols and formation of detergent/IMP was quasi-athermal, emphasizing the non-demicellization of the amphipols and the formation of mixed micelles, which found to be athermal (Diab *et al.*, 2007). We have also tested the ability of the APol/IMP complexes to resist extensive dilution (up to 140 times). The results showed that association of IMPs with amphipols, as expected, decreases drastically their dissociation rate, while diluting them in surfactant-free buffer. The robust nature found for the complexes, in the presence or absence of competing detergent, offer new insights into the behavior of aqueous solutions of Apol/IMP complexes, directly relevant to their use in biochemical or biophysical applications.

5.8. Conclusion

In this study, phosphorylcholine based amphiphilic polymers, with controlled architecture and good hydrophilic/lipophilic balance, were prepared and tested for the solubilization and stabilization of integral membrane proteins. This research project has succeeded in enhancing the aqueous solubility of IMPs by reaching high solubilization capacity, and consequently in increasing their stability at various pH, in solution of high salinity and upon extensive dilution. To the best of our knowledge, this is the first amphipols that could provide IMPs complexes with such stability. Phosphorylcholine based amphiphilic polymers offer new opportunities for the solubilization of IMPs namely because of: (i) their small sizes similar to that of the surfactant, (ii) their zwitterionic nature, which allows them to be stable in different media, (iii) their high solubilization capacity, (iv) their expected biocompatibility because phosphorylcholine is a good mimic of phosphatidylcholine, which comprises a large proportion of lipid membrane in organisms.

The need to detailed knowledge of the function of membrane proteins and their ability to facilitate membrane processes is essential to an understanding of drug delivery mechanisms, since to reach the site of action many drugs must be transported across the plasma membranes of cells either through the lipid bilayer or by interaction with membrane transporters and receptors. The results obtained in this research will aid to solve and understand many IMPs structure and functions, with the aim of designing new drugs for many non-curable diseases.

5.9. References

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CHAPTER SIX

Summary and perspectives

Integral membrane proteins and their complexes play crucial roles in many cellular and physiological processes. They are essential mediators of materials, information, and energy transfer between cells and their environment, between compartments within cells, and between compartments comprising the organ systems. They are now the targets for a large number of pharmacologically and toxicologically active substances and are responsible, in part, for their uptake, metabolism, and clearance. Functionally normal membrane proteins are vital to health, and specific defects are associated with many known disease states. An increase in the number of known membrane protein structures will contribute to an enhanced understanding of many basic phenomena underlying cellular functions mentioned above, which are essential to human health. However, development of biochemical assays with integral membrane proteins presents significant hurdles due to protein instability and loss of activity. This thesis describes the development of a phosphorylcholine based amphiphilic polymer, which resembles the native membrane environment and aids in the solubilization of integral membrane proteins while preserving their biological activity.

The present study has increased our understanding of the effect of chemical structure of amphiphilic copolymers on their efficiency to solubilize integral membrane protein and to keep them stable over extended period of time at different solution conditions. It also enhances the current understanding of amphiphilic polymer/neutral detergent systems and of the factors affecting protein stability, which will help to optimize the outcome of current membrane protein purification protocols and to better formulate polymer/surfactant systems used in pharmaceutics, cosmetics and foodstuffs. The information given in this thesis suggests that PC-amphipols provide an interesting new way of handling membrane protein in vitro. One of the advantages of PC-based amphipols derives from the possibility of working at acidic pH, in the presence of salts, and in the absence of detergent.

Amphipols trapped integral membrane proteins, being stable in the absence of detergent, can be used to study interactions with ligands and other macromolecules in the absence of complicating factor. A promising area of development is the study of the interactions of soluble factors with immobilized amphipols trapped membrane proteins, where the solution that is flown over the chip or resin does not needs to contain any surfactant. Amphipols can probably be used, among other macromolecules, to deliver membrane proteins and other molecules into the membrane of living cells without killing them, and to stabilize immunogenic preparations, as well as to detect, in detergent-free systems, antibodies directed against membrane proteins. Applications of these polymers to membrane protein structure analysis form an attractive field. For example, in solution-state NMR, where accelerating the tumbling rate of molecules is one of the keys to obtaining well- resolved spectra.

Even though data obtained are promising, further aspects must be examined before starting their applications. First, the toxicity of the polymer needs to be investigated *in vitro*. This study is of high importance if the polymer to be used in delivering membrane proteins to the membrane. Second, the preparation of PC-based amphipols where the phosphorylcholine is linked to the polymer backbone via amide bond. This will offer a PC-based amphiphilic polymer without net charge that prevails over the limitations of the first generation of PC- based amphipols.

The information gathered in this thesis is a step towards the obtention of a rich membrane protein data base. It seems that the use of polymers as solubilizers for integral membrane protein remains a field rich in opportunities, with many interesting aspects to discover.

Appendix A. Supplementary data

Complexation of Integral Membrane Proteins by Phosphorylcholine-Based Amphipols

C. Diab, C. Tribet, Y. Gohon, J.-L. Popot, F. M. Winnik

A.1. Raw data corresponding to the traces represented in Figure 2.7.

Note that the scale of the ordinates is the same in all plots to facilitate comparisons.

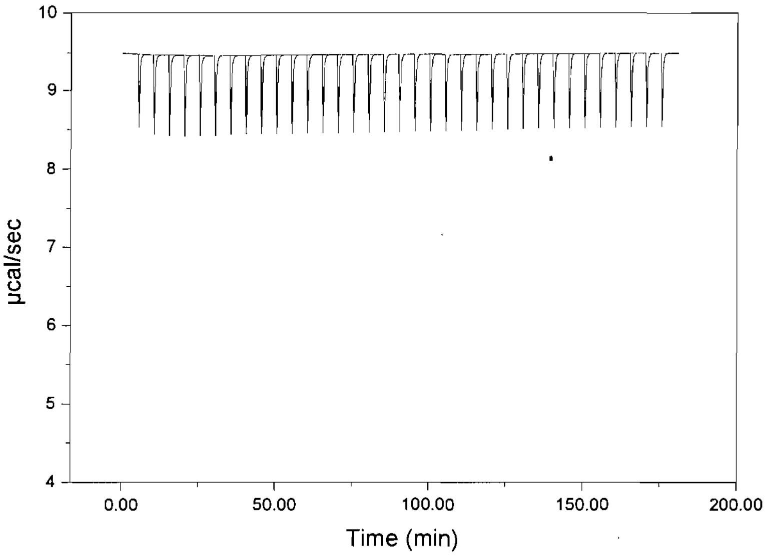

Figure 2.1.S: Calorimetric trace of the titration of an OTG solution (13.5 mM) into a solution of A8-35 (1 g/L).

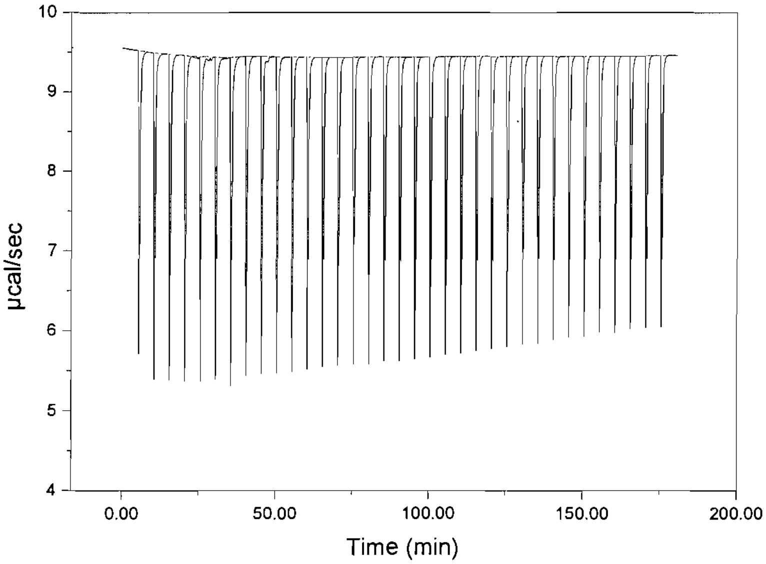

Figure 2.2S: Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of A8-35 (1 g/L).

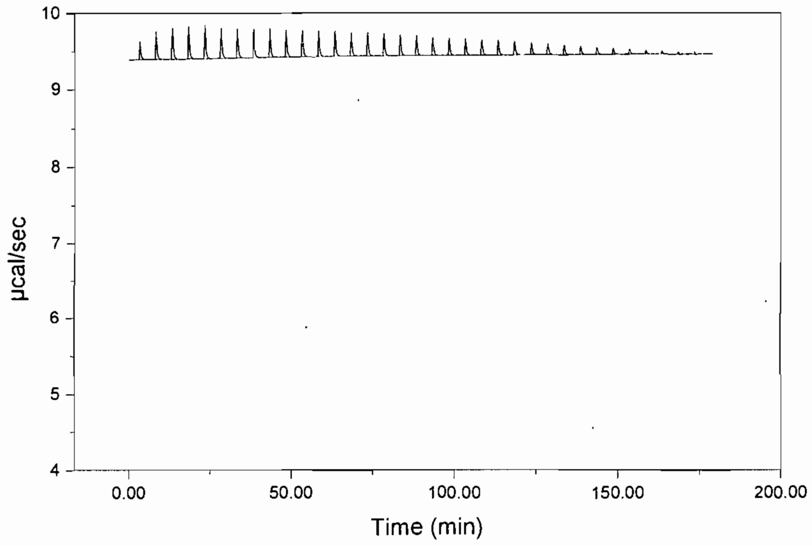

Figure 2.3S: Calorimetric trace of the titration of a solution of OTG (13.5 mM) into a solution of A8-35 (5 g/L).

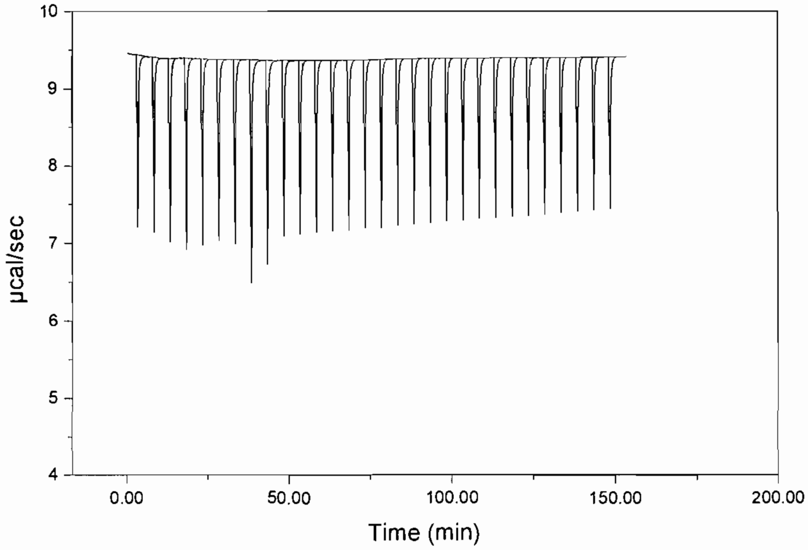

Figure 2.4S: Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of A8-35 (5 g/L).

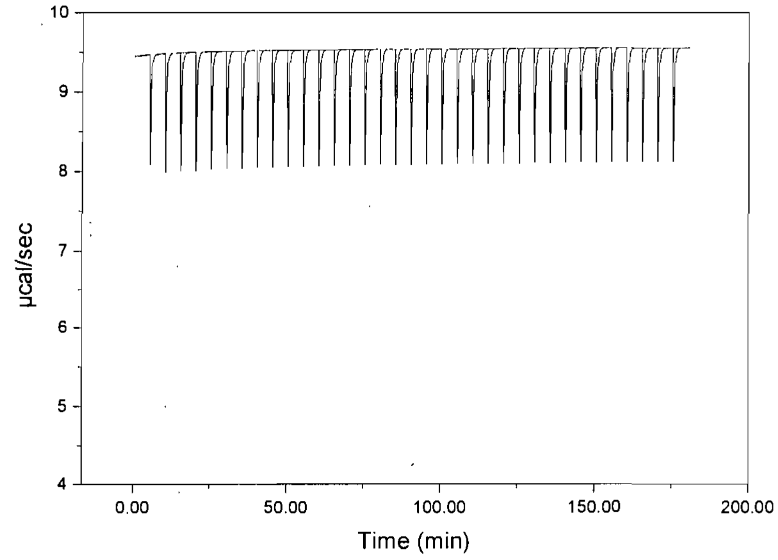

Figure 2.5S: Calorimetric trace of the titration of a solution of OTG (13.5 mM) into a solution of C22-43 (1 g/L).

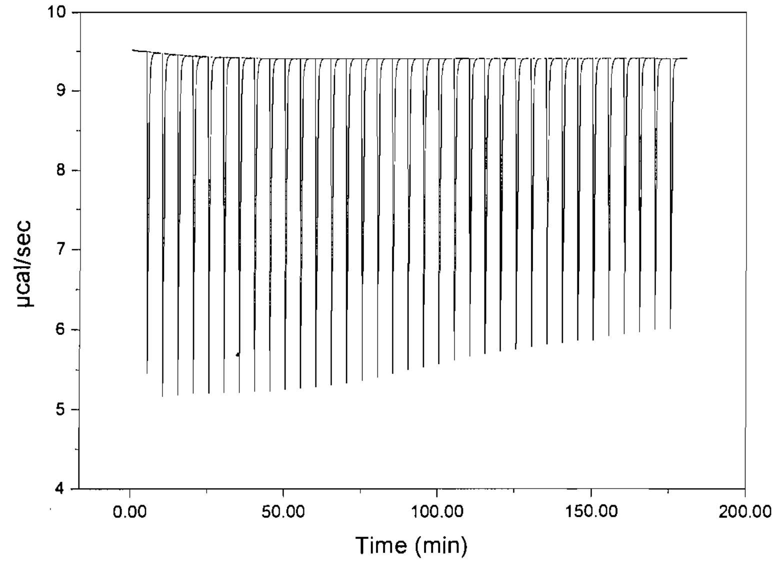

Figure 2.6S: Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of C22-43 (1 g/L).

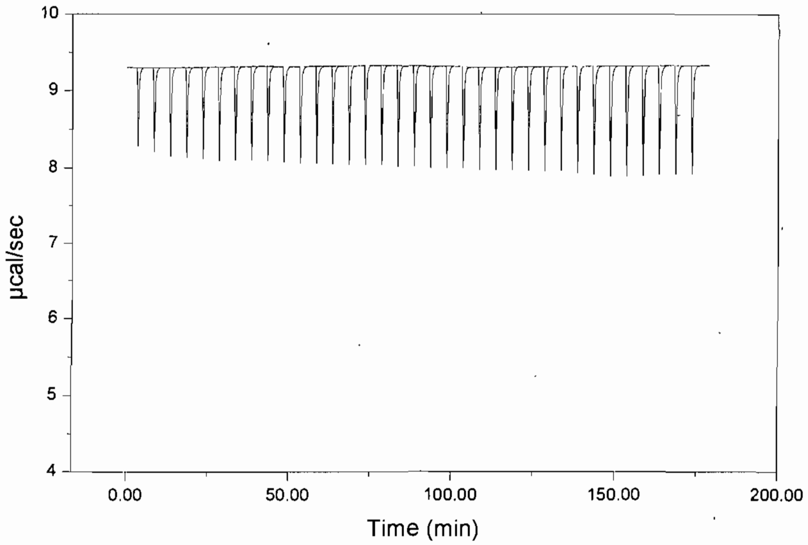

Figure 2.7S: Calorimetric trace of the titration of a solution of OTG (13.5 mM) into a solution of C22-43 (5 g/L).

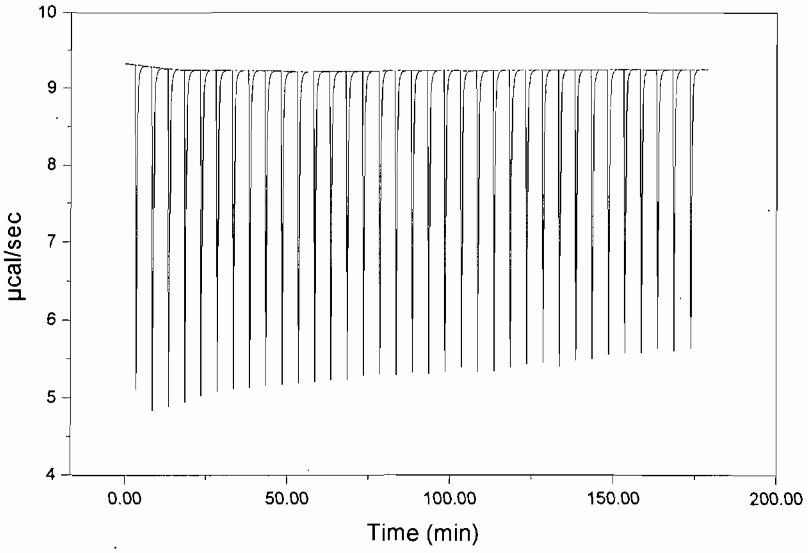

Figure 2.8S: Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of C22-43 (5 g/L).

A.2. Control experiments.

Two titrations were performed under experimental conditions identical to those described in the article to assess the effect of an imbalance of the buffer concentrations after dialysis on the results of the ITC experiments. Assuming that the error upon addition of NaCl to the solution after dialysis was 2% or less, we performed two control experiments: in one measurement, a buffer solution containing 100 mM NaCl was titrated into the identical buffer; in another titration, a buffer solution containing 95 mM NaCl was titrated into a buffer containing 100 mM NaCl. The enthalpograms obtained in each case are presented below. It can be observed that the difference in enthalpy in the latter case is ~22 J/mol, a negligeable value as compared to the enthalphies of interactions between amphipols and BR (Figure 2.7).

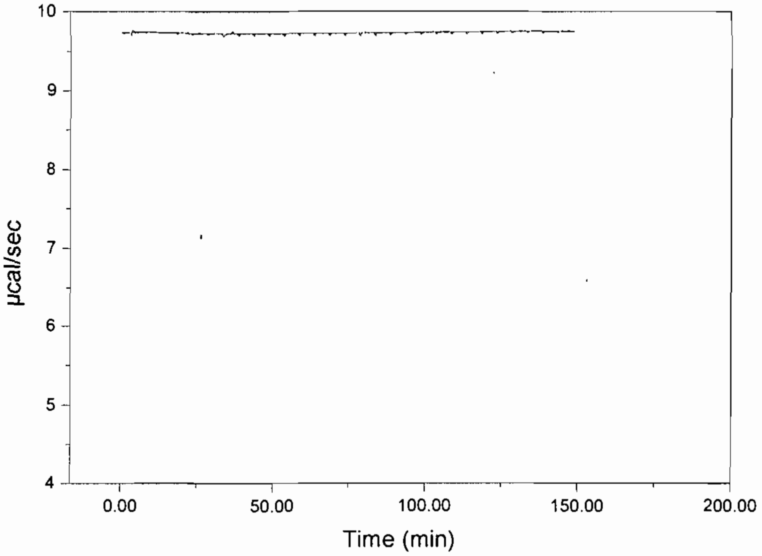

Figure 2.9S: Calorimetric trace of the titration of a buffer solution containing 100 mM of NaCl into the same buffer.

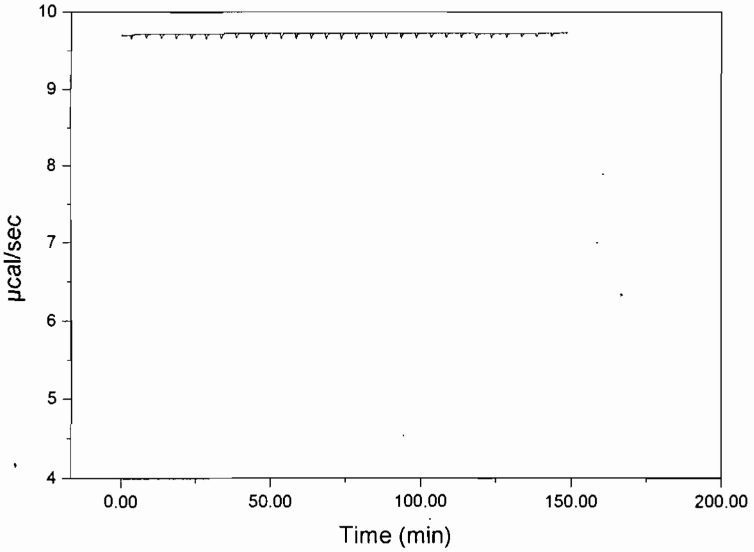

Figure 2.10S: Calorimetric trace of the titration of a buffer solution containing 95 mM of NaCl into the same buffer containing 100 mM of NaCl.

Appendix B. Supporting information

Enthalpy of Interaction and Binding Isotherms of Non-ionic Surfactants onto Micellar Amphiphilic Polymers (Amphipols).

C. Diab, F. M. Winnik and C. Tribet

B.1. Synthesis and characterization of the polymers C22-43 and C45-68

B.1.1. Materials

Azobisisobutyronitrile (AIBN, 98%) was recrystallized from methanol. *N*-isopropylacrylamide (stabilized, 99%) was obtained from Acros Organics and was recrystallized from acetone/heptane (4:6). The chain transfer agent, S-1-isobutyl-S'-(α,ά-dimethyl-α"-*N*-isopropylacetamide)trithiocarbonate (Kujawa *et al.*, 2006) and the monomers n-octylacrylamide (Effing *et al.*, 1994) and *N*-t-BOC-*N*-(ethylenedioxybis(ethyl)acrylamide (Miyazawa & Winnik, 2002) were prepared as described previously. 1,4-Dioxane (ACP, ACS Reagent) was distilled over sodium under argon. All other solvents were reagent grade and used as received.

B.1.2. Methods

Proton NMR spectra were recorded on a Bruker AMX-400 (400 MHz). The chemical shifts are referenced to trimethylsilane (TMS). UV/Visible spectra were measured with an Agilent 8452A photodiode array spectrometer. Infrared spectra were recorded on a Bruker Vector 22 equipped with a Harrick MVP ATR cell. Gel permeation chromatography (GPC) was performed with a GPC system consisting of an Agilent 1100 isocratic pump, a set of TSK-gel α-M and a TSK-gel α-3000 (Tosoh Biosep) columns, a Dawn EOS multi-angle laser light scattering detector (Wyatt Technology Corp.) and an Optilab DSP interferometric refractometer (Wyatt Technology Corp.); injection volume: 100 μL; flow rate: 0.5 mL min^{-1}; eluent:

DMF; temperature: 40 °C. The d*n*/dc values were measured with an Optilab DSP interferometric refractometer (Wyatt Technology Corp) used off-line.

B.1.3. Preparation of C22-43

The polymerization was performed in a septum-capped 25 mm x 150 mm glass tube placed in a First Mate system from Argonaut Technologies. A solution of NIPAM (0.25 g, 2.2 mmol), *N*-t-BOC-*N*-[(ethylenedioxybis(ethyl)acrylamide] (1.34 g, 4.4 mmol), *n*-octylacrylamide (0.4 g, 2.2 mmol) and S-1-isobutyl-S'-(α,ά-dimethyl-α"-*N*-isopropylacetamide)trithiocarbonate (26 mg, 0.0887 mmol) in dioxane (10 mL) was degassed for 30 minutes by vigorously bubbling nitrogen. The mixture was heated to 65 °C. Azobis(isobutyronitrile) (AIBN) (2.88 mg, 0.0175 mmol) was added at once. The polymerization mixture was stirred at 65 °C for 17 hr. It was cooled to room temperature. The solvent was removed by evaporation. The polymer formed was purified by two precipitations from THF into hexanes and dried in vacuo; ^{1}H NMR ($CDCl_3$, δ ppm): 0.88 (3H, $\mathbf{H_3}CC_7H_{14}$-), 1.16 (6H, $(\mathbf{CH_3})_2CH$-), 1.27 (12H, $-NHCH_2(\mathbf{CH_2})_6CH_3$), 1.44 (9 H, $(\mathbf{CH_3})_3CO$), 1.89 (2H, BOC-$NHC\mathbf{H_2}$-), 3.67 (8 H, CH_2 of the ethylenedioxy group), 4.0 (1 H, $(CH_3)_2C\mathbf{H}NHCO$-], 5.4 (1 H, -**NH**-). The molar mass of this polymer was obtained by GPC analysis (see experimental). The dn/dc value of this polymer in DMF at 40 °C was 0.088 cm^3/g at 690 nm.

The *N*-t-BOC protecting group was removed with trifluoroacetic acid followed by ion exchange with a Dowex 2X8-400 as described previously (Miyazawa & Winnik, 2002). Phosphorylcholine groups were linked to the polymer by reductive amination of phosphorylcholine glyceraldehydes (Miyazawa & Winnik, 2002) following a protocol described previously (Miyazawa & Winnik, 2002), yielding the polymer C22-43 which was purified by dialysis against water. It was isolated by lyophilization. ^{1}H NMR (CD_3OD, δ ppm): 0.92 (3H, $\mathbf{H_3}CC_7H_{14}$–), 1.16 (6H, $(C\mathbf{H_3})_2CH$–), 1.27 (12H, –$NHCH_2(C\mathbf{H_2})_6CH_3$), 2.88-3.2 (4H, –$CH_2C\mathbf{H_2}NHC\mathbf{H_2}$–), 3.25 (9H, $(C\mathbf{H_3})_3N^+$–), 3.67 (8H, CH_2 of ethylenedioxy group), 3.9 (1H, $(CH_3)_2C\mathbf{H}$–), 4.0 (2H, –$POC\mathbf{H_2}CH_2NHCH_2$–), 4.3 [2H, $(CH_3)_3N^+CH_2C\mathbf{H_2}OP$–]; FTIR (ν, cm^{-1}): 1226 (ν_{as} PO_2^-), 966 (ν_{as} N–CH_3).

B.1.4. Preparation of C45-68

A solution of *N*-t-BOC-*N*-[(ethylenedioxybis(ethyl)-acrylamide] (0.831 g, 2.75 mnol) and *n*-octylacrylamide (0.168 g, 0.92 mmol) in dioxane (10 mL) was degassed for 30 minutes by vigorously bubbling of nitrogen through the solution. The mixture was heated to 65 °C. Azobis(isobutyronitrile) (4.6 mg) was added at once. The polymerization mixture was stirred at 65 °C for 17 hr. It was cooled to room temperature. The solvent was removed by evaporation, and the polymer was purified by two precipitations from THF into hexanes and dried in vacuo (1.075 g). ^{1}H NMR ($CDCl_3$, δ ppm): 0.88 (3H, $\mathbf{H_3}CC_7H_{14}$), 1.27 (12H, –$NHCH_2(C\mathbf{H_2})_6CH_3$), 1.44 (9H, $(C\mathbf{H_3})_3CO$), 1.89 (2H, BOC-$NHC\mathbf{H_2}$-), 3.67 (8H, CH_2 of ethylenedioxy group), 5.4 (1*H*, –N**H**–). The molar mass of this polymer was obtained by GPC analysis (see experimental). The dn/dc value of this polymer in DMF was 0.088 cm^3 g^{-1} at 690 nm.

Deprotection of the amine and reductive amination of phosphorylcholine were performed as described in the case of C22-43. The resulting polymer was purified by dialysis against water and isolated by lyophilization. ^{1}H NMR (CD_3OD, δ ppm): 0.92 (3H, $\mathbf{H_3}CC_7H_{14}$–), 1.27 (12H, $-NHCH_2(C\mathbf{H_2})_6CH_3$), 2.88-3.2 (4H, $-CH_2C\mathbf{H_2}NHC\mathbf{H_2}-$), 3.25 (9H, $(C\mathbf{H_3})_3N^+-$), 3.67 (8H, CH_2 of ethylene dioxy group), 4.0 (2H, $-POC\mathbf{H_2}CH_2NHCH_2-$), 4.3 [2H, $(CH_3)_3N^+CH_2C\mathbf{H_2}OP-$]; FTIR (ν, cm^{-1}): 1226 (ν_{as} PO_2^-), 966 (ν_{as} $N-CH_3$).

B.2. Changes of the hydronamic radius of OTG micelles as function of OTG concentration

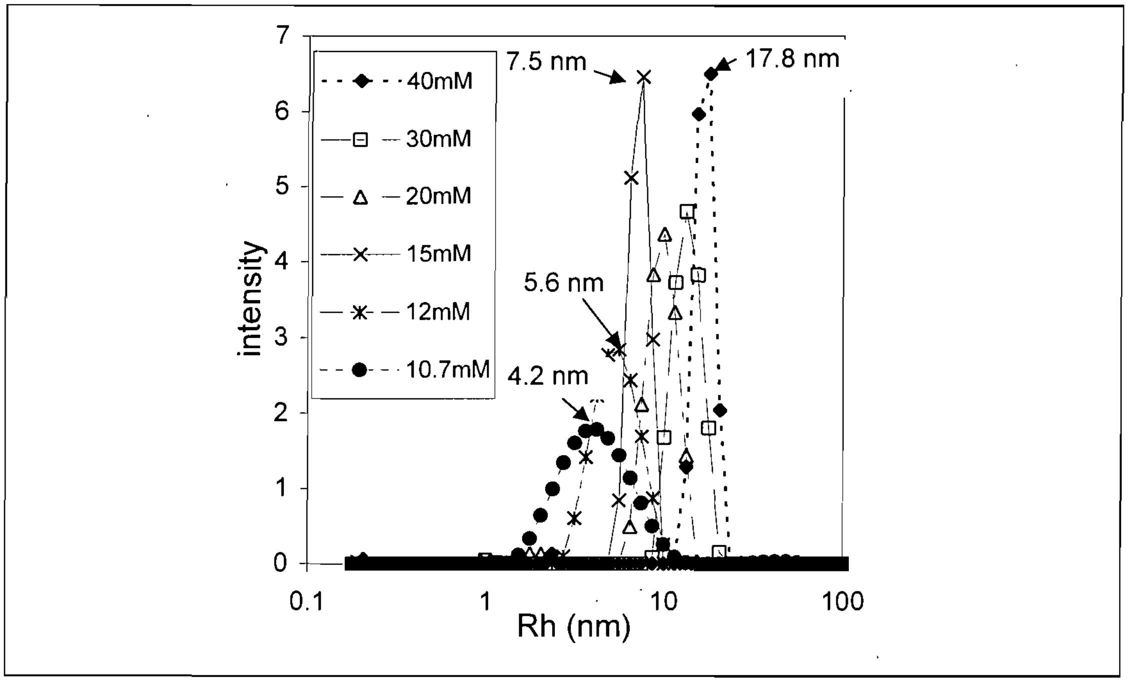

Figure 3.1S: Hydrodynamic radii distributions obtained from DLS measurements carried out with solutions of OTG in 20 mM NaOH-phosphoric acid buffer, pH 7.0. The concentrations of OTG are listed in the figure.

B.3. Expression of the enthalpy of mixing

The experimental molar enthalpy measured by ITC upon injecting surfactant micelles into a micellar amphipol solution was expressed as the sum of terms corresponding to the micelle dilution, the disintegration of polymer and of surfactant micelles, and the formation of amphipol/surfactant associates from isolated amphipols and surfactant molecules, as stated in Equation S1, where $N_{s,\,tot}$ is the molar amount of total surfactant injected:

$$N_{s,\,tot}\,\Delta H_{\text{exp}} = \Delta H_{\text{micelle dilution}} + \Delta H_{\text{surfactant demicellization}} + \Delta H_{\text{monomer dilution}} + \Delta H_{\text{polymer dilution}} + \Delta H_{\text{polymer demicellization}} + \Delta H_{\text{mixed association}} \qquad \textbf{(S1)}$$

Among the various enthalpic contributions to ΔH_{exp}, one can neglect the enthalpy of dilution of molecules or micelles which, experimentally, were shown to be very small, compared to the other contributions. The term $\Delta H_{\text{surfactant demicellization}}$ is known experimentally from ITC titrations of surfactant micelles in buffer. Control ITC experiments involving titrations of micellar amphipols in buffer indicated that demicellization of the amphipol assemblies does not occur in solution of concentrations higher than 0.5g/L. The last term $\Delta H_{\text{mixed association}}$ which corresponds to the excess enthalpy of mixing when surfactant micelles are mixed with polymer micelles in a given ratio is given by Equation S2, which invokes the regular solution approximation:

$$\Delta H_{\text{mixed association}} = N\Delta H_{reg} = N[X_s X_p \beta RT + X_s \Delta H_{mic,s} + X_p \Delta H_{mic,p}] \qquad \textbf{(S2)}$$

where N is the number of moles of amphiphiles involved in the association, X_s and X_p = 1- X_s are the mole fractions of amphiphiles in the mixed associates, T is the temperature, R is the universal gas constant, $\Delta H_{\text{mic,s}}$ and $\Delta H_{\text{mic,p}}$ are the molar enthalpies of micellization of surfactant and polymer, respectively. From equations S1 and S2 and neglecting the enthalpies of dilution, one can write:

$$N_{s,tot}\Delta H_{\text{exp}} \sim -N_{mic,s}\Delta H_{mic,s} + (N_s + N_p)\Delta H_{reg} - N_p \Delta H_{mic,p} \qquad \textbf{(S3)}$$

where $N_{s,\ tot}$ and $N_{mic,s}$ are the molar amounts of total surfactant injected and of surfactant injected in the micellar form, respectively, N_s is the number of surfactant molecules that enter into associates, and N_p is the total molar amount of alkyl groups lined to the amphipols, $X_{\text{s}} = N_{\text{s}} /(N_{\text{p}}+N_{\text{s}})$ and $X_{\text{p}} = N_{\text{p}}/(N_{\text{s}} + N_{\text{p}})$. Assuming that all the alkyl groups belonging to the polymer chains enter in micelle formation, irrespective of the presence of detergent, the last term in equation S2 ($N. X\text{p}\ \Delta H_{\text{mic,p}}$) is exactly the opposite of the last term in equation S3 ($\Delta H_{\text{polymer demicellization}}$ = - $N_p\Delta H_{\text{mic,p}}$)

B.4. Changes of the calculated enthalpies for various pairs of A and β

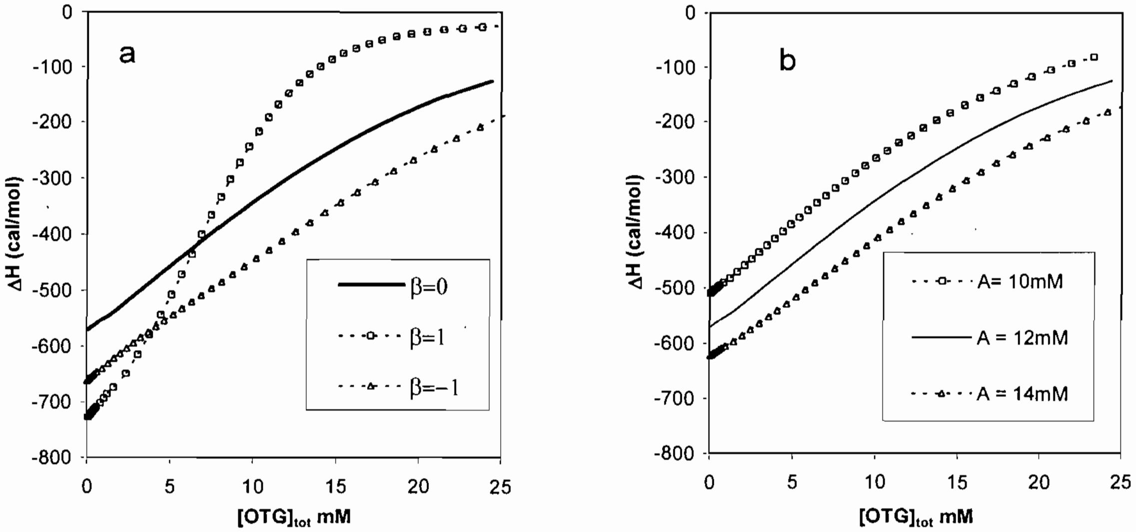

Figure 3.2S: Calculated enthalpy of mixing as a function of the total OTG concentration in the cell for addition of a 100 mM OTG solution in a solution of A8-35 (5 g/L). Data points were obtained from equations 3 and 5 as detailed in the text, with the values of the parameters A and β listed in the figure.

B.5. References

Effing, J. J., McLennan, I. J., van Os, N. M., Kwak, J. C. T. J. (1994) *^{1}H NMR investigations of the interactions between anionic surfactants and hydrophobically modified poly(acrylamide)s.* Phys. Chem. 98, 12397-12402.

Kujawa, P., Segui, F., Shaban, S., Diab, C., Okada, Y., Tanaka, F., Winnik, F. M. (2006) *Impact of end-group association and main-chain hydration on the thermosensitive properties of hydrophobically modified telechelic poly(N-isopropylacrylamides) in water.* Macromolecules 39, 341-348.

Miyazawa, K., Winnik, F. M. (2002) *Synthesis of phosphorylcholine-based hydrophobically modified polybetaines.* Macromolecules 35, 2440- 2444.