Towards a better understanding of the polyhydroxyalkanoate synthase from *Ralstonia eutropha*: Protein engineering and molecular biomimetics

A thesis presented to Massey University in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Microbiology

Anika Carolin Jahns

2009

With love and gratitude to

Mum & Dad

And in memory of my grandmother

Acknowledgement

I would like to thank my supervisor Prof Bernd H. A. Rehm for excellent guidance and support in the last three years, and for giving me the opportunity to come to New Zealand and gain insights into an interesting topic with a bright future.

Many thanks also to my co-supervisor Dr. Jan Schmidt who did not get tired of listening to all – crazy – ideas.

Massey University and especially IMBS have been great hosts. A big thank you to everyone. In the beginning it was hard to find my way around but there was always someone willing to lend me a hand – thank you.

I am grateful for the financial support, granted by the New Zealand Foundation for Research Science and Technology. There were a lot of reports involved but I think we managed quite well.

Furthermore, I would like to thank all members of the Rehmlab. We had a lot of fun during the last three years and certainly did not talk only about work. A special thanks to the PHA group. I will miss our Wednesday cake rounds. Katrin and Jane kept me good company in the middle lab and did not flee even when I broke into song. Thank you.

Coming to a foreign country where I did not know anybody and where people spoke a strange language, certainly not English (at least that is what I thought initially) was a big step. I met a lot of very interesting people along the way, always friendly and helpful.

I would especially like to thank Sophia Macris and Murray Scott for their friendship and support, for making me laugh and enjoy life outside university.
I am indebted to Sophia for everything, but especially for proof-reading my thesis and still being my friend afterwards.

Preface

This thesis is written according to the regulations of the latest version of the Handbook for Doctoral Studies, version 5, published by the Doctoral Research Committee in January 2009. The format of this thesis complies with the format of a thesis based on publications as described in the chapter "Submission of a thesis based on publications" on page 64.

All chapters that are published or submitted for publication are listed below. These contributions do not appear in chronological order.

Chapter I B

Indira A. Rasiah, Natalie Parlane, Katrin Grage, Rajasekaran Palanisamy, Anika C. Jahns, Jane A. Atwood and Bernd H. A. Rehm (2009). Biopolyester particles: preparation and applications. **Encyclopedia of Industrial Biotechnology**: in press

Chapter I C

Katrin Grage, Anika C. Jahns, Natalie Parlane, Rajasekaran Palanisamy, Indira A. Rasiah, Jane A. Atwood and Bernd H. A. Rehm (2009). Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/nano-beads in biotechnological and biomedical applications. **Biomacromolecules 10(4): 660-669**

Chapter II

Anika C. Jahns and Bernd H. A. Rehm (2009). The class I polyhydroxyalkanoate synthase from *Ralstonia eutropha* tolerates translational fusions to its C terminus: A new mode of functional display. **Applied and Environmental Microbiology**: in press

Chapter III

Anika C. Jahns, Richard G. Haverkamp and Bernd H. A. Rehm (2008). Multifunctional Inorganic-Binding Beads Self-Assembled Inside Engineered Bacteria. **Bioconjugate Chemistry 19(10):** 2072-2080

Chapter IV

Anika C. Jahns, Verena Peters and Bernd H. A. Rehm (2009). Engineering of bacterial polyester inclusions towards the display of ten lysine residues and potential applications. **Journal of Biomedicine and Biotechnology** – submitted for publication (2. Submission in revised form)

Listed below are all research contributions to the chapters/publications performed by Anika Jahns:

Chapter I B: The review was partly written by Anika Jahns, focussing on the background description and particle formation of polymalate and the applications of PHA particles.

Chapter I C: The parts of the review article describing the phasins and the regulatory proteins were contributed by Anika Jahns.

Chapter II: All experiments were performed by Anika Jahns. Verena Peters is acknowledged for constructing the plasmid $pCWE_{Spe}$-Mpl-EC.

Chapter III: Except for AFM measurements, all experiments were performed by Anika Jahns. Richard G. Haverkamp obtained all AFM data.

Chapter IV: Verena Peters constructed the plasmids pHAS+phaPwt and pHAS+phaPpolylys. All further experiments regarding the granule isolation, characterization and identification of the respective proteins and the silica binding assays were performed by Anika Jahns.

DNA sequencing, MALDI-TOF/MS, GC/MS and TEM analyses were provided by external services.

This is to certify that the above mentioned research has been conducted by Anika Jahns.

(Date, Signature)   (Date, Signature)
Prof. Bernd H. A. Rehm   Anika Jahns

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters composed of *(R)*-3-hydroxy-fatty acids. A variety of gram-positive as well as gram-negative bacteria and some archaea are able to produce these biopolymers as energy and carbon storage materials. In times of unbalanced growth, when carbon is available in excess but other nutrients are limited, PHA inclusions are formed. These granules are water-insoluble, stored intracellularly and can be maintained outside the cell as beads. The key enzyme for the formation of PHA inclusions is the PHA synthase PhaC, which catalyses the polymerization of *(R)*-3-hydroxyacyl-CoA to PHA with the concomitant release of CoA.

The PHA synthase from *Ralstonia eutropha* (currently *Cupriavidus necator*), which is covalently bound to the PHA granule surface, tolerates fusions to its N terminus without loss of activity. In this study it was investigated if it would also tolerate translational fusions to its C terminus. A specially designed linker was employed, aiming at maintaining the hydrophobic surroundings of the *R. eutropha* synthase C terminus to allow proper folding and activity. Two reporter proteins were tested as fusion partners, the maltose binding protein MalE and the green fluorescent protein GFP. As GFP is a hydrophobic protein itself, no additional linker between the PHA synthase and the reporter protein was necessary to produce PHA granules displaying the functional fusion protein on the surface. Principally, the PHA synthase PhaC tolerates translational fusions to its C terminus but the nature of the fusion partner influences the functionality.

Recently, PHA granules have often been acknowledged as bio-beads. A one-step production allows the formation of functionalised beads without the need for further cross-linking to impart desired surface properties. PHA beads displaying a gold- or silica-binding peptide at the N terminus of PhaC were constructed and tested for their applicability. Additionally, these beads were able to bind IgG due to the ZZ domain of the IgG binding protein A, which was employed as a linker sequence. These functionalised beads can be used as molecular tools in bioimaging and biomedicine, combining organic core with inorganic-binding shell structures.

In a different biomimetic approach, the display of ten lysine residues at the granule surface was achieved using the phasin protein PhaP as the anchoring matrix. Extensive work was performed in an attempt to also employ the synthase protein, but was unsuccessful. These positively charged bio-beads can be used for dispersion or cross-linking experiments as well as silica binding.

Table of Contents

Chapter I

Chapter I A

Chapter I B

Chapter I C

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

List of Figures

List of Tables

Abbreviations

AFM	Atomic Force Microscopy
Au	Gold (aurum)
BSA	Bovine serum albumin
˚C	Degrees Celcius
CoA	Coenzyme A
d	Density
DEAE	Diethylaminoethyl cellulose
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
Fig.	Figure
GAP	Granule associated protein
GC	Gas chromatography
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
kDa	Kilo Dalton

LacZ	β-Galactosidase
LB	Luria-Bertani
M	Molar (mol/l)
MALDI-TOF	Matrix-assisted laser desorption ionisation/time-of-flight
MalE	Maltose binding protein
MS	Mass spectrometry
Mw	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
PLA	Polylactide
PLGA	Poly(lactic-co-glycolic acid)
PMLA	Poly(β-L-malate)
SDS	Sodium dodecyl sulphate
TEM	Transmission Electron Microscopy

Chapter I A

Introduction

Natural Polyesters

Natural Polyesters

Three different organic polyesters are naturally produced on earth. Polymalic acid is only produced in eukaryotic microorganisms. Cutin and suberin are polymer matrices for lipophilic cell wall barriers in plants and polyoxoesters are produced in prokaryotic bacteria and archaea. All these polyesters are formed naturally without any outside intervention, e.g. genetic engineering, and are therefore investigated for their potential to be exploited, improved or modified to be applicable in modern technology.

Polymalic Acid

Malic acid is an organic compound with the formula $HO_2CCH_2CHOHCO_2H$. L-malic acid units are covalently linked by ester bonds between the hydroxyl group and the carboxyl group in the β position to form poly(β-L-malic acid). The carboxyl group in the α position points away from the polyester chain (Fischer *et al.*, 1989). The ionized form of the polymer is poly(β-L-malate) or PMLA and is highly water soluble. This carboxylic-functionalised polyester can be produced by either chemical synthesis or fermentation of myxomycetes and certain filamentous fungi (Lee *et al.*, 2002). Natural production of poly(malic acid) was first reported for *Penicillium cyclopium* where it inhibits acid proteases (Shimada and Matsushi, 1969; Shimada and Matsushima, 1967; Shimada *et al.*, 1969). Some work has been published regarding its production in *Aureobasidium pullulans* (Liu and Steinbüchel, 1996; Liu and Steinbüchel, 1997; Nagata *et al.*, 1993) but most of the studies were performed with *Physarum polycephalum* (Fischer *et al.*, 1989; Göttler and Holler, 2006; Korherr *et al.*, 1995; Lee *et al.*, 2002; Rathberger *et al.*, 1999). The slime mould *Physarum polycephalum* differentiates into several cell forms during its life cycle (Burland *et al.*, 1993) but only the plasmodium, a giant polynuclear cell, produces PMLA. There it accumulates to high concentrations comparable to the level of DNA in the naturally synchronous nuclei (Angerer and Holler, 1995; Fischer *et al.*, 1989; Schmidt *et al.*, 1996). All myxomycetes species examined so far contained PMLA in their plasmodia. The polymer level in the nuclei remains constantly high during the cell cycle; excess amounts of PMLA are secreted into the culture medium. The unique physiological role of this unconventional biopolymer is currently being investigated for the plasmodium of *Polycephalum* and although the underlying biochemical connections are not fully

understood, it appears that PMLA functions as a storage and carrier molecule to maintain an even distribution of histones, DNA polymerases and other nuclear proteins within this giant cell (Angerer and Holler, 1995; Doerhoefer *et al.*, 1998). Furthermore, it has been assumed that by assuring an equal distribution of nuclear proteins, PMLA is involved in the coordination of the synchronous replication of DNA during the cell cycle (Fischer *et al.*, 1989). Poly(β-L-malate) was discovered for its ability to bind and reversibly inactivate DNA polymerase α (Fischer *et al.*, 1989; Holler *et al.*, 1992). Later it was shown that DNA polymerases δ and ε could also be bound and inactivated by PMLA whereas DNA polymerase β was not inhibited (Achhammer *et al.*, 1995; Doerhoefer *et al.*, 1998; Doerhoefer *et al.*, 2002). The distance between the negative charges in PMLA is responsible for the specificity of the binding and inactivation. This distance is comparable to that between the phosphate groups in the DNA backbone, explaining the competitive binding of PMLA and DNA to the polymerases (Holler *et al.*, 1992). Because DNA polymerase α of *Physarum polycephalum* is constitutively expressed during the cell cycle (Fischer, 1987; MacNicol *et al.*, 1987) and histones are only synthesized during the S phase, it was proposed that PMLA, histones, polymerase and newly replicated DNA interact competitively during the cell cycle with the DNA polymerase being active during the S phase and inactive during the G2 phase. Following the hypothesis, histone synthesis starts at the beginning of the S phase (Carrino *et al.*, 1987) and the newly formed histones compete with the DNA polymerase α for PMLA binding and eventually succeed. The displaced polymerase starts DNA replication until the histone synthesis ends and the histone proteins are used to form energetically highly favourable nucleosomes with the newly replicated DNA. Free PMLA again binds DNA polymerase α – which marks the beginning of the G2 phase – and DNA synthesis is therefore terminated (Holler *et al.*, 1992).

PMLA is constitutively formed in the plasmodium (Schmidt *et al.*, 1996) and NMR studies revealed that different biosynthesis routes are employed, depending on the nutrients available (Lee and Holler, 1999; Lee *et al.*, 1999). Recently, the polymer has also been investigated regarding its use in biotechnology. It is available from sustainable feedstock which can be maintained with renewable resources (Holler 1997) and is biodegradable and biocompatible. PMLA is a bioresorbable compound and gets metabolized in the mammalian tricarboxylic acid cycle (Braud *et al.*, 1985; Fournie *et al.*, 1990; Vert and Lenz, 1979). In biomedicine PMLA is investigated as a non-toxic, nonimmunogenic drug delivery system especially for antitumor applications

(Abdellaoui *et al.*, 1998; Fujita *et al.*, 2007; Heun *et al.*, 1999; Lee et al., 2006; Ljubimova *et al.*, 2008; Mueller *et al.*, 2008; Portilla-Arias *et al.*, 2008).

Cutin and Suberin

Life on earth originated in water but more than 400 million years ago the first plants moved from a wet environment onto land (Kenrick and Crane, 1997). In order to survive in the dryer surroundings, plants developed a protective barrier, known as a "cuticle". Cuticles cover all air exposed exteriors of plants and are composite materials, basically made of cutin (Kolattukudy, 1980, 2001). Cutin is an insoluble polymer, consisting of hydroxy-fatty acids and their derivatives linked by ester bonds. The most common monomers in cutin are 16-hydroxypalmitate and 9,16 or 10,16-dihydroxypalmitate and 18-hydroxyoleate as well as 9,10-epoxy-18-hydroxystearate and 9,10,18-trihydroxystearate. These monomers are produced and modified in the epidermal cells of plants (Kolattukudy, 2001). There is not much known about the concrete structure of cutin, whether it is a distinct polymer of unknown molecular weight, possibly anchored to the cell wall at a few points, or whether it is a highly cross-linked continuum (Pollard *et al.*, 2008). As cutin is part of the water-resistant protective outside layer, it is presumed to participate in controlling non-stomatal water and gas exchange and host-plant interactions (Kolattukudy, 2001; Nawrath, 2006). It has also been proposed that cutin is involved in plant morphology (Hoffmann-Benning and Kende, 1994) and in controlling organ identity by counteracting organ fusion (Sieber *et al.*, 2000).

The other type of polyester found in plants is suberin, the main constituent of cork, named after the cork oak *Quercus suber* L. (Graca and Pereira, 2000b). Suberin is a waxy substance, highly hydrophobic and consists of two domains: polyaromatic and polyaliphatic (Bernards, 2002; Kolattukudy, 2001). The aromatic domain is a polyphenolic substance, derived from cinnamic acid and its derivatives, mostly ferulic acid. The aliphatic polyester domains are composed of C16 and C18 fatty acids and their elongation products, similar to those of cutin (Bernards and Lewis, 1998; Bernards *et al.*, 1995; Bernards and Razem, 2001; Gandini *et al.*, 2006; Kolattukudy, 2001). It was shown previously that glycerol is part of suberin (Kolattukudy, 1980; Kolattukudy, 2001; Kolattukudy and Espelie, 1989), but only recently it was detected to be an essential building block (Bernards and Lewis, 1998; Graca and Pereira, 1997; Graca and Pereira, 1999; Graca and Pereira, 2000a, b, c). There is no "unique" chemical

structure of suberin and over the years numerous models have been proposed (Gil *et al.*, 1997; Lopes *et al.*, 2000; Sitte, 1962). In recent years newly developed methods allowed first insights into linkages within the polymer and the following model was designed: From glycerol as a basis, suberin grows two- and three-dimensionally due to the formation of ester linkages between monomers of the aliphatic domain. At the periphery of this glycerol-based polymer, ferulic-acid covalently links the aliphatic polyester to the aromatic domain, which itself is thought to form a connection to the carbohydrates of the cell wall (Franke and Schreiber, 2007; Kolattukudy, 1977, 1981; Lopes *et al.*, 2000b). The main function of suberin is to protect against desiccation, which was essential when plants first moved from the wet to a dry environment. The most prominent example for suberin deposition in the cell wall is the Casparian strip or Casparian band in endodermal root cells. This structure prevents the water and nutrients taken up by the roots from moving through the apoplast, instead, they must pass the cell membrane and traverse through the symplast. This selection tool is mostly determined by the aliphatic domains of the polymer (Hose *et al.*, 2001; Zimmermann *et al.*, 2000). Mangroves use suberin to minimize salt intake from their habitat. The importance of suberin in salt stress tolerance was also shown for *Arabidopsis* (Beisson *et al.*, 2007). Suberin is also synthesized as wound response, where healthy tissue is protected by suberin accumulation at the wound edges (Kolattukudy, 2001). The fact that suberin deposition occurs only in specific tissues and in response to environmental changes indicates a complex regulation of the polymer synthesis for which the molecular processes are so far unknown (Franke and Schreiber, 2007).

Bacterial Polyoxoesters

Polyhydroxyalkanoates (PHAs) were discovered at the beginning of the 20th century, when Lemoigne first found poly(3-hydroxybutyrate) (PHB) inside bacterial cells (Lemoigne, 1926). PHAs are biopolyesters synthesized by many bacteria and some archaea in phases of carbon excess but limitation of other nutrients (Hoffmann and Rehm, 2005; Kim *et al.*, 2004; Kuchta *et al.*, 2007). The polyesters are stored in the form of water-insoluble spherical inclusions or granules inside the cell and serve as energy and carbon storage (Rehm, 2006). Approximately 150 different hydroxyalkanoic acids are known as constituents of PHAs (Steinbüchel and Valentin, 1995) and the most investigated PHA is poly(3-hydroxybutyrate), mainly studied in *Cupriavidus necator* (formerly *Ralstonia eutropha*) (Peoples and Sinskey, 1989; Slater

et al., 1988). Recently, PHB and related co-polymers attracted interest because of their thermoplastic and elastomeric properties and biodegradability, and are being thoroughly investigated regarding their qualification to replace petroleum-derived plastics. Additionally, several biomedical and biotechnological applications have been investigated and assessed (Grage *et al.*, 2009; Philip *et al.*, 2007). Intracellularly formed PHA granules can be stably maintained outside the cell as PHA beads. The following two chapters provide further detailed insights into the applications of PHA and especially PHA beads. Also, current knowledge of the emergence and formation of PHA granules as well as a short description of associated proteins will be presented.

References

Abdellaoui K, Boustta M, Vert M, Morjani H, Manfait M. 1998. Metabolite-derived artificial polymers designed for drug targeting, cell penetration and bioresorption. Eur. J. Pharm. Sci. 6:61-73.

Angerer B, Holler E. 1995. Large complexes of beta-poly(L-malate) with DNA polymerase alpha, histones, and other proteins in nuclei of growing plasmodia of *Physarum polycephalum*. Biochemistry 34:14741-14751.

Achhammer G, Winkler A, Angerer B, Holler E. 1995. DNA polymerase delta of *Physarum polycephalum.* Curr. Genet. 28:534-45.

Beisson F, Li Y, Bonaventure G, Pollard M, Ohlrogge JB. 2007. The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of *Arabidopsis*. Plant Cell 19:351-368.

Bernards MA, Lopez ML, Zajicek J, Lewis NG. 1995. Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin. J. Biol. Chem. 270:7382-7386.

Bernards MA, Lewis NG. 1998. The macromolecular aromatic domain in suberized tissue: a changing paradigm. Phytochemistry 47:915-933.

Bernards MA, Razem FA. 2001. The poly(phenolic) domain of potato suberin: a non-lignin cell wall bio-polymer. Phytochemistry 57:1115-1122.

Bernards MA. 2002. Demystifying suberin. Canadian Journal of Botany-Revue Canadienne De Botanique 80:227-240.

Braud C, Bunel C, Vert M. 1985. Poly(beta-malic acid) - A new polymeric drug-carrier - Evidence for degradation *in vitro*. Polymer Bulletin 13:293-299.

Burland TG, Solnica-Krezel L, Bailey J, Cunningham DB, Dove WF. 1993. Patterns of inheritance, development and the mitotic cycle in the protist *Physarum polycephalum*. Adv. Microb. Physiol. 35:1-69.

Carrino JJ, Kueng V, Braun R, Laffler TG. 1987. Distinct replication-independent and - dependent phases of histone gene expression during the *Physarum* cell cycle. Mol. Cell. Biol. 7:1933-1937.

Doerhoefer S, Khodyreva S, Safronov IV, Wlassoff WA, Anarbaev R, Lavrik OI, Holler E. 1998. Molecular constituents of the replication apparatus in the plasmodium of *Physarum polycephalum*: identification by photoaffinity labelling. Microbiology 144 (Pt 11):3181-3193.

Doerhoefer S, Windisch C, Angerer B, Lavrik OI, Lee BS, Holler E. 2002. The DNA-polymerase inhibiting activity of poly(beta-l-malic acid) in nuclear extract during the cell cycle of *Physarum polycephalum*. Eur. J. Biochem. 269:1253-1258.

Fischer H. 1987. Universität Regensburg.

Fischer H, Erdmann S, Holler E. 1989. An unusual polyanion from *Physarum polycephalum* that inhibits homologous DNA polymerase alpha in vitro. Biochemistry 28:5219-5226.

Fournie P, Domurado D, Guerin P, Braud C, Vert M, Madelmont JC. 1990. In vivo fate of end-chain radiolabeled poly(beta-malic acid), a water-soluble biodegradable drug carrier. J. Bioact. Compat. Polym. 5:381-395.

Franke R, Schreiber L. 2007. Suberin--a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. 10:252-259.

Fujita M, Lee BS, Khazenzon NM, Penichet ML, Wawrowsky KA, Patil R, Ding H, Holler E, Black KL, Ljubimova JY. 2007. Brain tumor tandem targeting using a combination of monoclonal antibodies attached to biopoly(beta-L-malic acid). J. Controlled Release 122:356-363.

Gandini A, Pascoal C, Silvestre AJD. 2006. Suberin: A promising renewable resource for novel macromolecular materials. Prog. Polym. Sci. 31:878-892.

Gil AM, Lopes M, Rocha J, Pascoal Neto C. 1997. A 13C solid state nuclear magnetic resonance spectroscopic study of cork cell wall structure: the effect of suberin removal. Int. J. Biol. Macromol. 20:293-305.

Göttler T, Holler E. 2006. Screening for beta-poly(L-malate) binding proteins by affinity chromatography. Biochem. Biophys. Res. Commun. 341:1119-1127.

Graca J, Pereira H. 1997. Cork suberin: A glyceryl based polyester. Holzforschung 51:225-234.

Graca J, Pereira H. 1999. Glyceryl-acyl and aryl-acyl dimers in *Pseudotsuga menziesii* bark suberin. Holzforschung 53:397-402.

Graca J, Pereira H. 2000a. Diglycerol alkenedioates in suberin: building units of a poly(acylglycerol) polyester. Biomacromolecules 1:519-522.

Graca J, Pereira H. 2000b. Methanolysis of bark suberins: Analysis of glycerol and acid monomers. Phytochem. Anal. 11:45-51.

Graca J, Pereira H. 2000c. Suberin structure in potato periderm: glycerol, long-chain monomers, and glyceryl and feruloyl dimers. J. Agric. Food Chem. 48:5476-5483.

Grage K, Peters V, Palanisamy R, Rehm BHA. 2009. Polyhydroxyalkanoates: from bacterial storage compound via alternative plastic to bio-bead. In: Rehm BHA, editor. Microbial production of biopolymers and polymer precursors: Applications and perspectives. Norfol, UK: Horizon Bioscience.

Heun G, Lambov N, Zlatkov A, Peikov P, Doytchinova I, Gesheva K. 1999. Biodegradable cross-linked prodrug of the bronchial dilator Vephylline. 2. Kinetics and quantum chemical studies on the release mechanism. J. Controlled Release 58:189-194.

Hoffmann-Benning S, Kende H. 1994. Cuticle Biosynthesis in Rapidly Growing Internodes of Deepwater Rice. Plant Physiol. 104:719-723.

Hoffmann N, Rehm BHA. 2005. Nitrogen-dependent regulation of medium-chain length polyhydroxyalkanoate biosynthesis genes in pseudomonads. Biotechnol. Lett. 27:279-282.

Holler E. 1997. Poly(malic acid) from natural sources. In: Cheremisinof NP, editor. Handbook of Engineering Polymeric Materials. New York: Marcel Dekker. p 93-103.

Holler E, Achhammer G, Angerer B, Gantz B, Hambach C, Reisner H, Seidel B, Weber C, Windisch C, Braud C and others. 1992. Specific inhibition of *Physarum polycephalum* DNA-polymerase-alpha-primase by poly(L-malate) and related polyanions. Eur. J. Biochem. 206:1-6.

Hose E, Clarkson DT, Steudle E, Schreiber L, Hartung W. 2001. The exodermis: a variable apoplastic barrier. J. Exp. Bot. 52:2245-2264.

Kenrick P, Crane PR. 1997. The origin and early evolution of plants on land. Nature 389:33-39.

Kim YR, Paik HJ, Ober CK, Coates GW, Batt CA. 2004. Enzymatic surface-initiated polymerization: a novel approach for the in situ solid-phase synthesis of biocompatible polymer poly(3-hydroxybutyrate). Biomacromolecules 5:889-894.

Kolattukudy PE. 1977. Lipid polymers and associated phenols, their chemistry, biosynthesis, and role in pathogenesis. In: Loewus FA, editor. Recent Advances in Phytochemistry: Runeckles VC Plenum Publishing. p 185-246.

Kolattukudy PE. 1980. Biopolyester Membranes of Plants: Cutin and Suberin. Science 208:990-1000.

Kolattukudy PE. 1981. Structure, biosynthesis, and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. Plant Mol. Biol. 32:539-567.

Kolattukudy PE. 2001. Polyesters in higher plants. Adv. Biochem. Eng. Biotechnol. 71:1-49.

Kolattukudy PE. 2001. Suberin from plants. In: Doi Y, Steinbüchel A, editors. Biopolymers: Wiley. p 41-68.

Kolattukudy PE, Espelie KE. 1989. Chemistry, biochemistry, and function of suberin and associated waxes. In: Rowe J, editor. Natural products of woody plants, chemical extraneous to the lignocellulosic cell wall. Berlin, Heidelberg: Springer. p 304-367.

Korherr C, Roth M, Holler E. 1995. Poly(beta-L-malate) hydrolase from plasmodia of *Physarum polycephalum*. Can. J. Microbiol. 41:192-199.

Kuchta K, Chi L, Fuchs H, Pötter M, Steinbüchel A. 2007. Studies on the influence of phasins on accumulation and degradation of PHB and nanostructure of PHB granules in *Ralstonia eutropha* H16. Biomacromolecules 8:657-662.

Lee BS, Holler E. 1999. Effects of culture conditions on beta-poly(L-malate) production by *Physarum polycephalum*. Appl. Microbiol. Biotechnol. 51:647-652.

Lee BS, Maurer T, Kalbitzer HR, Holler E. 1999. beta-Poly(L-malate) production by *Physarum polycephalum* - C-13 Nuclear magnetic resonance stwdies. Appl. Microbiol. Biotechnol. 52:415-420.

Lee BS, Vert M, Holler E. 2002. Water-soluble aliphatic polyesters: poly(malic acid)s. In: Doi Y, Steinbüchel A, editors. Biopolymers. Weinheim: Wiley-VCH. p 75-103.

Lee BS, Fujita M, Khazenzon NM, Wawrowsky KA, Wachsmann-Hogiu S, Farkas DL, Black KL, Ljubimova JY, Holler E. 2006. Polycefin, a new prototype of a multifunctional nanoconjugate based on poly(beta-L-malic acid) for drug delivery. Bioconjugate Chem. 17:317-326.

Lemoigne M. 1926. Produits de dehydration et de polymerisation de l'acide beta-oxobutyrique. Bull. Soc. Chim. Biol. 8:770-782.

Liu S, Steinbüchel A. 1996. Investigation of poly(beta-L-melic acid) production by strains of *Aureobasidium pullulans*. Appl. Microbiol. Biotechnol. 46:273-278.

Liu S, Steinbüchel A. 1997. Production of poly(malic acid) from different carbon sources and its regulation in *Aureobasidium pullulans*. Biotechnol. Lett. 19:11-14.

Ljubimova JY, Fujita M, Khazenzon NM, Lee BS, Wachsmann-Hogiu S, Farkas DL, Black KL, Holler E. 2008. Nanoconjugate based on polymalic acid for tumor targeting. Chem. Biol. Interact. 171:195-203.

Lopes MH, Gil AM, Silvestre AJ, Neto CP. 2000. Composition of suberin extracted upon gradual alkaline methanolysis of *Quercus suber* L. cork. J. Agric. Food Chem. 48:383-391.

Lopes MH, Neto CP, Barros AS, Rutledge D, Delgadillo I, Gil AM. 2000. Quantitation of aliphatic suberin in *Quercus suber* L. cork by FTIR spectroscopy and solid-state (13)C-NMR spectroscopy. Biopolymers 57:344-351.

MacNicol AM, Banks GR, Cox RA. 1987. Biosynthesis and activity of DNA polymerase throughout the mitotic cycle of *Physarum polycephalum*. FEBS Lett. 221:48-54.

Mueller W, Haindl M, Holler E. 2008. *Physarum* polymalic acid hydrolase: Recombinant expression and enzyme activation. Biochem. Biophys. Res. Commun. 377:735-740.

Nagata N, Nakahara T, Tabuchi T. 1993. Fermentative production of poly(beta-L-malic acid), a polyelectrolytic biopolyester, by *Aureobasidium* sp. Biosci. Biotechnol. Biochem. 57:638-642.

Nawrath C. 2006. Unraveling the complex network of cuticular structure and function. Curr. Opin. Plant Biol. 9:281-287.

Peoples OP, Sinskey AJ. 1989. Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. 264:15298-15303.

Philip S, Keshavarz T, Roy I. 2007. Polyhydroxyalkanoates:biodegradable polymers with a range of applications. J. Chem. Technol. Biotechnol. 82:233-247.

Pollard M, Beisson F, Li Y, Ohlrogge JB. 2008. Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci. 13:236-246.

Portilla-Arias JA, Garcia-Alvarez M, de Ilarduya AM, Holler E, Galbis JA, Munoz-Guerra S. 2008. Synthesis, degradability, and drug releasing properties of methyl esters of fungal poly(beta,L-malic acid). Macromol. Biosci. 8:540-550.

Rathberger K, Reisner H, Willibald B, Molitoris HP, Holler E. 1999. Comparative synthesis and hydrolytic degradation of poly (L-malate) by myxomycetes and fungi. Mycol. Res. 103:513-520.

Rehm BHA. 2006. Genetics and Biochemistry of Polyhydroxyalkanoate Granule Self-assembly: The Key Role of Polyester Synthases. Biotechnol. Lett. 28:207-213.

Schmidt A, Windisch C, Holler E. 1996. Nuclear accumulation and homeostasis of the unusual polymer beta-poly (L-malate) in plasmodia of *Physarum polycephalum.* Eur. J. Cell Biol. 70:373-380.

Shimada K, Matsushima K. 1967. Nippon Nogeikagaku Kaishi 41:454-458.

Shimada K, Matsushima K. 1969. A protease inhibitor from *Penicillium cyclopium.* I. Purification and partial characterization. Agric. Biol. Chem. 33:544-548.

Shimada K, Matsushima K, Fukumoto J, Yamamoto T. 1969. Poly-(L)-malic acid; a new protease inhibitor from *Penicillium cyclopium.* Biochem. Biophys. Res. Commun. 35:619-624.

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Metraux JP, Nawrath C. 2000. Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12:721-738.

Sitte P. 1962. Zum Feinbau der Suberinschichten im Flaschenkork. Protoplasma 54:555-559.

Slater SC, Voige WH, Dennis DE. 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-beta-hydroxybutyrate biosynthetic pathway. J. Bacteriol. 170:4431-4436.

Steinbüchel A, Valentin HE. 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128:219-228.

Vert M, Lenz RW. 1979. Preparations and properties of poly-beta-malic acid: a functional polyester of potential biomedical importance. ACS Polymer Preprints 20:608-611.

Zimmermann HM, Hartmann K, Schreiber L, Steudle E. 2000. Chemical composition of apoplastic transport barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). Planta 210:302-311.

Chapter I B

Introduction

Biopolyester particles: preparation and applications

I. A. Rasiah[1], N. Parlane[2], K. Grage[1], R. Palanisamy[1], A. C. Jahns[1], J. A. Atwood[1] and B. H. A. Rehm[1,*]

[1]Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand, and [2]Hopkirk Research Institute, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand

* Corresponding author: Bernd H. A. Rehm, e-mail: b.rehm@massey.ac.nz,

phone: +64 6 350 5515 ext. 7890, fax: +64 6 350 5688

Biopolyester particles: preparation and applications

I. A. Rasiah, N. Parlane, K. Grage, R. Palanisamy, A. C. Jahns, J. A. Atwood and

B. H. A. Rehm

Abstract

Over the past few decades, there has been a growing awareness of the potential uses of biodegradable polymers in biotechnology and medicine. In particular, micro- and nano-sized particles which are made from natural resources, exhibiting biodegradability, biocompatibility and low toxicity have been the subject of intense research and development in many areas. Apart from their beneficial material properties, these particles allow variations in structure and composition to be designed, thereby tailoring them for specific applications. This review presents a broad overview of the main categories of biopolyester particles described in the literature, with emphasis on the composition and preparation of each group, followed by the wide variety of applications and potential uses described to date.

Introduction

Biopolyester particles are micro- and nanometer sized particles made from natural and renewable resources. In the past few decades, great advances have been made in the use of such particles throughout various fields of science and medicine, mainly due to their properties such as biodegradability and biocompatibility (1, 2). In particular, the area of drug delivery has been highly impacted with the range of polymeric biomaterials which have been processed into micro- and nano-spheres as well as capsules with the specific purpose of encapsulating or attaching pharmacologically active substances (3). The main goal in the design of such particles has been the controlled release of a bioactive agent to a specific site at a therapeutically optimal rate (4).

Several categories of biodegradable polymer particles have been conceived for various applications. These include (i) naturally occurring particles such as the polyhydroxyalkanoate (PHA) granules produced intracellularly by many bacteria (5, 6), (ii) particles which are chemically synthesized from extracted PHA (7), (iii) micro- and

nano-particles based on polylactide (PLA) (3), (iv) co-polymers of PLA with glycolic acid, such as PLGA (8) and particles produced from polymalate (poly(β-malic acid), PMLA (9) (Figure 1). While these categories are by no means exclusive, they represent the major types of biotechnologically relevant as well as long-circulating, particulate carriers which have now taken a leading position in drug formulation and delivery.
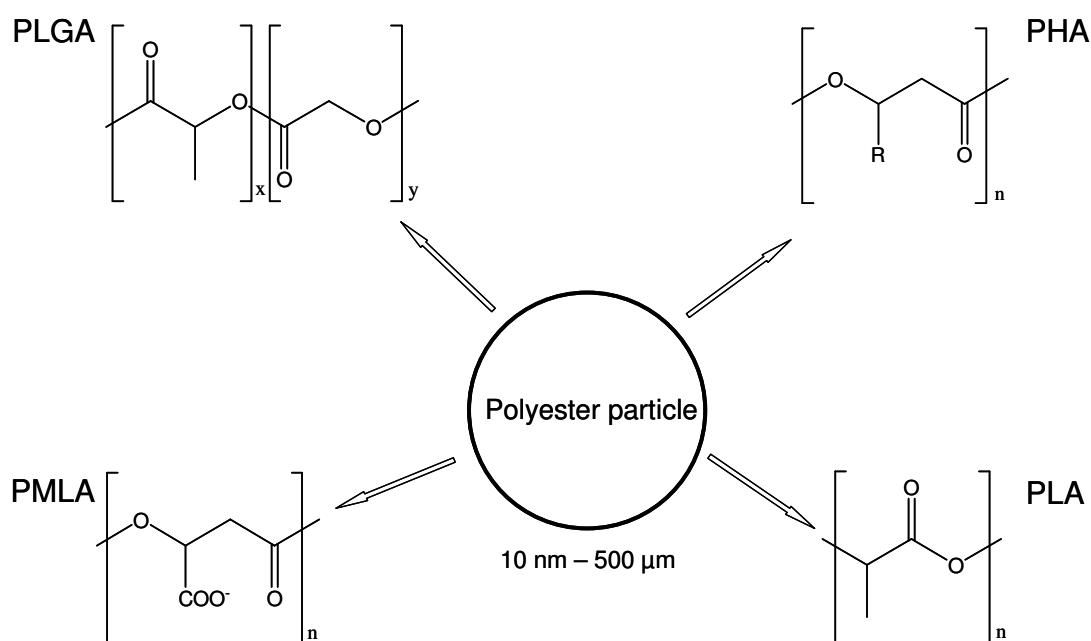
Figure 1: Overview of different biopolyester constituents

The use of particles as carriers has several advantages over traditional oral and intravenous methods of drug administration, including high stability, ability to carry and administer several drugs at the same time at a specific site and the possibility of several routes of administration, such as oral or topical administration as well as inhalation (10). As vaccine delivery systems, the advantages include the administration of multiple antigens in a one-off immunization allowing the simultaneous uptake of various antigens by the respective immune cells, protection of the antigen *in vivo* and control over the rate of release (11, 12).

One of the fundamental requirements of particles used in therapeutic applications is biodegradation of the polymer within the human body. PLA and PLGA degrade *in vivo* by hydrolysis into lactic acid and lactic acid plus glycolic acids, respectively, which get incorporated into the cellular tricarboxylic acid cycle and hence oxidised (13). Similarly, PMLA is degradable by simple hydrolysis of the ester bond of the main chain, yielding malic acid which is in turn completely biodegraded to carbon dioxide

and water (14). The degradation behaviour of these polymer materials has been the subject of much research (1, 15, 16), especially with regard to the mechanisms of degradation (14) and factors affecting the degradation processes *in vivo* (17, 18).

In addition to the growing number of medical applications, particles derived from biological polymers are well known for their use in biotechnological settings. In particular, particles made of the PHA, polyhydroxybutyrate (PHB), have been widely investigated. These include (i) particles which have been synthesized from extracted PHA (19, 20) and (ii) granules which are intracellularly formed in recombinant bacteria and subsequently isolated (6). PHA particles have been successfully engineered to functionally display specific biotechnologically relevant fusion proteins on their surface such as streptavidin (21), binding peptides for inorganic materials (22) an antibody (23) and to immobilize a thermostable α-amylase (24). These novel, functionalized beads are considered to be valuable tools for various applications in biomedicine and industry. This review provides an overview of the main categories of biodegradable polyester particles used in biotechnological and therapeutic applications. Each type of particle, the composition of the respective biopolymer and the preparative methods for particle formation will be discussed. Finally, attention will be drawn to the wide variety of biomedical applications of each group, thus highlighting the level of progress achieved in this area in the past few decades.

Polyhydroxyalkanoates

Background

PHAs are bacterial storage compounds which are formed in times of imbalanced nutrient availability when a carbon source is available in excess but other nutrients have been depleted. They are deposited as water-insoluble spherical inclusions or PHA granules inside the cell and can be mobilized when conditions are suitable. PHA accumulation is quite common among both Gram-negative and Gram-positive bacteria and has even been shown to occur in some members of the *Archaea* (25-30). Depending on the synthesizing organism (and to some extent on the substrate) PHAs are composed of monomers of different chain lengths and have been divided into short chain length (PHA$_{SCL}$) and medium chain length PHAs (PHA$_{MCL}$). PHA$_{SCL}$ is mainly found as poly(3-hydroxybutyric acid) (PHB). While physical and thermal properties of

the different PHAs vary, they are all biocompatible and biodegradable (31, 32). There is considerable interest in applications of PHA, but due to the relatively high production costs compared to oil-based plastics, PHAs are currently mainly attractive for use in the medical field, e.g. for sutures or implants like heart valves (33, 34).

PHB biosynthesis has been extensively investigated in the Gram-negative bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) where it involves three steps starting from acetyl-coenzyme A (CoA) (35-37). Two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by the enzyme β-ketothiolase (PhaA). Acetoacetyl-CoA reductase (PhaB) reduces acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA and these hydroxyacyl-CoA monomers are subsequently polymerized by PHB synthase (PhaC). The PHA synthase is the key enzyme of PHA biosynthesis in general; for PHAs other than PHB different hydroxyacyl-CoA percursors derived from different metabolic pathways are used. Nascent polyester chains assemble to form the PHA granule, with the PHA synthase remaining covalently bound to the polyester molecule and thus immobilized at the granule surface (38, 39). Other proteins that associate with the granule surface in a non-covalent manner are PHA depolymerising enzymes (PhaZ in *C. necator*), regulatory proteins (PhaR in *C. necator*) and structural phasins (PhaP in *C. necator*) (40-50).

PHAs can be recombinantly produced e.g. in *Escherichia coli* by heterologous expression of the required PHB biosynthesis genes while providing appropriate cultivation conditions (51-54). PHA production in plants has also been considered (55, 56). Recently, native PHA granules and *in vitro* synthesized PHA beads have been increasingly considered for applications as functionalized micro- or nanobeads in biotechnology and biomedicine (57).

Preparation of PHA particles

PHA micro- and nanoparticles can be prepared chemically/*in vitro* from extracted PHA or be produced *in vivo* inside the (mainly bacterial) cell and subsequently isolated. In both cases a protein of interest can be immobilized at the bead surface through fusion to a granule-associated protein. However, in case of *in vitro* synthesized beads proteins are also bound *in vitro* to pre-fabricated beads, whereas protein binding to *in vivo* produced beads occurs during PHA granule formation inside the cell. *In vitro* bead preparation is usually the method of choice for drug loading/encapsulation.

In vitro production

As PHA has long been considered as an alternative plastic, a range of methods have been developed for the extraction of PHA from cells, the aim being the isolation of the material and not the preservation of its spherical nature / granule shape (58-60). Extraction is usually preceded by biomass harvest and, depending on the method, cell disruption. A common method to isolate PHA from the biomass is solvent-based extraction. Cells (generally lyophilized) are resuspended in a solvent such as chloroform or methylene chloride in which PHA is soluble (61, 62). After all other cell debris has been removed, for example by filtration, the PHA can be precipitated with methanol or ethanol. Non-chlorinated solvents such as acetone have also been used, however, acetone only solubilises PHA_{MCL}, not PHB (63). While solvent-based extraction yields PHA of high purity without affecting molecular weight, it is necessary to use large volumes of solvent, which is expensive and might also be undesirable for other reasons. Water-based extraction methods comprise digestion and/or solubilization of non-PHA cellular material. Hypochlorites, in general sodium hypochlorite, have been used in this method and were found to enable relatively cheap extraction of very pure PHA, but do significantly degrade PHA in the process (64, 65). The combined use of sodium hypochlorite and chloroform was reported to reduce degradation while still enabling isolation of high purity PHA (66, 67). Similarly, hypochlorite has also been used in combination with surfactants (68). Several other protocols have been developed which use different combinations of detergents and/or enzymes to solubilise cell components (69-71). Extraction of PHA with supercritical fluids, mainly supercritical carbon dioxide, has been reported to be particularly effective for removing lipid contaminants, resulting in extremely pure PHA containing only low levels of endotoxin (72).

Starting from purified PHA, micro- or nanoparticles for subsequent functionalization can be prepared by variants of the oil-in-water emulsion method (7). Lee and coworkers (73) and Wang and coworkers (74) used slightly varied versions of the oil-in-water emulsion method to generate microbeads from PHB and nanobeads from poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx), respectively. In both cases, the polymer was dissolved in chloroform and an aqueous solution with or without surfactant added. The separate phases were emulsified with a homogenizer or by sonication, and PHB or PHBHHx beads harvested by evaporation of the chloroform and/or centrifugation, followed by repeated washing. According to Horowitz *et al.,* the

addition of surfactant results in the formation of beads containing amorphous PHA, which crystallizes upon removal of the surfactant, i.e. by washing with water (7). Omission of the surfactant leads to crystallization of the PHA as soon as the chloroform evaporates.

Yao and coworkers generated PHBHHx nanoparticles loaded with the lipid dye rhodamine B isothiocyanate (RBITC) by a modified oil-in-water solvent diffusion technique (75). RBITC and PHBHHx were dissolved in dichloromethane and the dichloromethane mixture was slowly added to a sonicated poly(vinyl alcohol) solution. After further sonication and extended gentle stirring, the particles were collected by centrifugation and subsequently washed in water.

For drug delivery, microcapsules and microspheres of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) containing different chemicals/drugs have been prepared by slightly varied solvent emulsion/evaporation techniques, all using dichloromethane as solvent and poly(vinyl alcohol) as emulsifier (76, 77). In addition, double emulsification methods (water-in-oil-in-water and oil-in-water-in-oil) were developed (78), and different copolymer compositions and molecular weights were investigated (79, 80). The primary aim of this research was to understand how the polymer properties and the preparation technique (81) influence the physical properties of the resulting microspheres as well as drug loading and release (see below).

In vivo production

In order to exploit the advantage of having proteins naturally immobilized at the bead surface, it is possible to use *in vivo* produced PHA granules as functionalized micro-/nanobeads. In this case, isolation conditions have to be gentler to preserve granule shape/properties and protein functionality. Production generally happens in recombinant strains, not necessarily natural PHA producers, but this does not impact on the (common) isolation methods. These methods usually involve a form of density gradient centrifugation, using either a glycerol or a sucrose gradient.

In earlier publications, native PHA granules were prepared by repeated centrifugation of cell lysates layered onto glycerol, using swing-out rotors (82, 83). Much purer granule preparations were obtained when glycerol gradients, consisting of four layers with glycerol concentrations between 10.5 and 9.0 M, centrifuged at 90,000 x g were used for further purification (38). Alternatively, glycerol gradient centrifugation can be performed with only two layers of 88% and 44% (v/v) glycerol at 100,000 x g (84).

Both linear and discontinuous sucrose gradients have been used for the isolation of native PHA granules (85-87). Preparation of a linear gradient can be a complex procedure (88), but it has also been generated without the need for a special apparatus by freezing a discontinuous gradient (consisting of 2 M, 1.66 M, 1.33 M and 1 M sucrose layers) in liquid nitrogen and then thawing it at 4°C (87). Alternatively, PHA granules have been isolated on a discontinuous sucrose gradient consisting of two layers of 20% and 15% (w/w) sucrose (86). In both cases centrifugation was done at 110,000 x *g*.

For some applications of functionalized PHA beads in protein purification, no prior isolation of the beads is required (see below) (89, 90)

Applications of PHA particles

PHA beads, naturally or artificially derived, are very versatile and can be used for a broad range of applications, mostly in biotechnology and biomedicine. Over the years, different strategies of how the PHA system can be used for protein purification have been evolved. All these different approaches have focussed on efficiency of purification and low costs compared to commercially available chromatography systems. In the recently published applications the granule-associated phasins were employed as the basis for the purification of the desired proteins. In a very simple approach, the N-terminal granule binding domain of PhaF, a phasin from *Pseudomonas putida* GPo1 (48), was used as a binding/purification tag called BioF (91). The protein of interest was co-expressed as a fusion protein with the BioF purification tag under PHA accumulating conditions. Thus the protein of interest was produced bound to PHA granules and could be released by mild detergent treatment. The released protein still contained the purification tag (91). Further developed methods have used inteins for facilitated release of the target protein (19). Inteins, or INTervening protEINS, are naturally occurring protein sequences that are capable of post-translational self-excision from a precursor protein, employing a process called splicing (19). Proteins of interest are fused via an intein linker to the phasin protein PhaP and therefore attached to the natively formed granules inside the cell. For better attachment, multiple copies of PhaP were employed. The protein of interest is released using the intein cleaving site. This system was successfully established in the natural PHA producer *C. necator* as well as in engineered *E. coli.* This does not necessarily require isolation of the engineered particles (89, 90), because the crude cell extract containing the particles can be directly

used for protein purification. Recently, a slightly modified purification method was published. It is still based on the PhaP-intein-X fusion, with X being the protein of interest, but it separates the protein production from the actual purification process, resembling common chromatography techniques. The PHA particles which serve as purification matrix are artificially (*in vitro*) made out of chemically purified PHA. The crude extract of the recombinantly produced PhaP-tagged protein is then incubated with the artificially prepared matrix. PhaP mediated binding and subsequent intein cleavage leads to purification of the protein (92). A completely different approach was employed by Brockelbank *et al.* (2006), in which the ZZ domain of protein A from *Staphylococcus aureus* was fused to the PHA synthase PhaC, enabling affinity purification of antibodies of the IgG class (93). These engineered beads performed equally when compared to commercially available protein A beads. In this last example the protein to be purified did not need a special purification tag, whereas in all other methods the protein of interest was tagged with a granule-binding protein to enable attachment to the purification matrix.

Micro- and nano-PHA beads displaying certain functionalities are very valuable and beneficial, especially for biotechnological applications. In most cases these beads are of non-biological origin and the production involves chemical cross-linking (20). Several studies have described the one-step production of functionalized PHA beads in engineered *E. coli*. In all examples the N terminus of the PHA synthase was fused to the respective protein that should be displayed at the bead surface, for example streptavidin (21) or *β*-galactosidase (21, 84) and *α*-amylase (24) as examples of immobilized enzymes. These beads are functional tools for various applications, ranging from bio-labelling using the strong binding between streptavidin and biotin to supplementing washing detergents with a starch hydrolysing enzyme that shows high temperature stability.

Because of its biodegradable and biocompatible properties, PHA is being investigated for its applicability in biomedicine. Immunoassays are an especially widely used tool. For these assays, the proteins must be in a functional state, but immobilized. These criteria can be easily fulfilled using PHA beads. *E. coli* has been engineered to display the PhaP-bound mouse interleukin-2 (IL2) or the myelin oligodendrocyte glycoprotein (MOG) on PHA granules (5). These eukaryotic proteins were functional albeit their production in a prokaryotic host and performed successfully in fluorescence activated cell sorting (FACS). The PHA beads were produced in one step. An enterokinase site

between the PhaP protein and the respective fusion partner allowed cleavage of the fusion partner and could hence be used for protein purification. In a related study it was shown that two independent functionalities can be displayed at the PHA bead surface either in the form of two functionalized proteins or one bifunctional protein (94). Beads displaying the separate GFP-PhaC and PhaP-MOG fusion proteins, as well as the GFP-PhaP-MOG bifunctional fusion protein showed discrete signals in FACS analysis and no overlapping or interference could be observed. Lee *et al.* (2005) immobilized proteins on artificially derived PHA particles, using the substrate binding domain (SBD) of the PHA depolymerase (73). The SBD tagged proteins were produced in *E. coli* and then bound to the PHA particles in a way described previously for some of the protein purification methods (73). This method also resulted in functionally displayed proteins and FACS analysis could be successfully performed.

Biopolyester particles are already being used for applications in the delivery of drugs or similar compounds. PHA particles are just emerging in this area. PHA copolymers, especially PHBV, have been investigated with regard to encapsulation and release of different types of drugs (76, 77, 80, 81, 95-97). These studies have mainly focussed on the formation of drug-loaded particles, distinguishing between monolithic microspheres and reservoir-type microcapsules. Interestingly, stabilizers, e.g. gelatine, had a big influence on the drug-release properties of the particles. The dispersion of the drug in gelatine, followed by the encapsulation of the drug-gelatine mixture in PHBV proved to be disadvantageous for medical applications. These particles showed a triphasic release pattern, typically for microspheres, with alternating periods of slow and more rapid drug release (78, 97). The release properties could be changed to a zero order kinetics profile, when the drug was encapsulated in PHBV and the whole particle coated with gelatine. These particles showed a linear release profile over a period of 30 days, which would be more favourable for biomedical applications (78). Recently, Yao *et al.* (2008) (75) described the use of prefabricated PHA beads for targeted drug delivery to macrophages. The mannosylated human α1-acid glycoprotein and the human epidermal growth factor were attached to PHA particles by using PhaP as binding domain as described above. To mimic the drug load the PHA particles were stained with a lipid-soluble dye. It was reported that macrophages recognized the attached proteins with their specific receptors. The particles were ingested by endocytosis, suggesting potential applications in targeted drug delivery.

Another biomedical application of PHA particles in the diagnostic area was recently described. Multifunctional PHA beads, displaying an IgG binding domain and a special peptide sequence for binding inorganics (gold or silica) were proposed as useful tools for bioimaging and diagnostics. It could be shown that both functionalities reacted independently from each other and no inhibition or interference could be detected. These beads allow the antibody based targeted delivery of a contrast agent to selected tissues (22). Furthermore, beads displaying the single-chain variable fragment (scFv) of an anti-β-galactosidase antibody were engineered, using the PHA synthase as anchoring motif (23). These functionalized beads were successfully used for the purification of β-galactosidase as an example of a custom-made high affinity purification matrix. Once again, formation of these beads resulted cost-effectively from a microbial one-step production process. The displayed antibody fragment was correctly folded and instantly active and did not require any additional cross-linking. These examples demonstrate the versatility of functionalized PHA beads either derived from extracted PHA or directly formed inside engineered microbial cells for applications in biotechnology and medicine.

Polylactides

Background

PLA, a biopolymer derived from lactic acid (2-hydroxypropionic acid), has been widely studied for its applications in the medical field and also for use in the food packaging industry. PLA is biodegradable (1), bioresorbable (3) and exhibits properties such as low immunogenicity and toxicity, which have led to FDA approval for its use in human injections (98). In addition, lactic acid-based polymers have tensile strength and rheological properties suitable for applications as packaging materials and food containers (99) as well as various films, fibres and homeware (100).

Lactic acid, the basic unit of PLA, is produced by microorganisms, animals and plants in nature (101). Lactic acid exists as two optically active forms, namely the L(+) and D(-) isomers. While both forms are produced by fermentation in bacteria, only the L(+) isomer is found in mammalian systems. Since lactic acid is present in the biochemical pathways of most living organisms, polymers based on this organic material will degrade to natural metabolic compounds (102, 103).

PLA is usually produced as a mixture between the isomeric forms and therefore the nature of the polymeric material formed varies depending on the ratio between these. The process of PLA formation is a stereocomplex crystallization or racemic crystallization and has been described by Tsuji (104). PLA made purely from L(+) lactide is called poly(L-lactide), or PLLA, while PDLA is the corresponding lactide made from D(-) lactide. PLLA is a semicrystalline powder with a melting temperature of about $180^{o}C$, whereas introduction of the D(-) isomer reduces the melting temperature and the degree and rate of crystallization (100). Thus, the degree of crystallinity determines the physical and mechanical properties of the polymer (105) as well as its degradation rate (106).

High molecular weight PLA (about 100,000 Daltons) can be produced by different routes (for schemes see Sodergard and Bhardwaj as well as Mohanty (101, 105)), namely (a) direct condensation polymerization which initially produces a low molecular weight pre-polymer that is then polymerized by chain coupling agents (105, 107, 108), (b) azeotrophic dehydrative condensation (99, 105) and (c) ring opening polymerization (ROP). The ROP route involves generation of the cyclic dimer lactide (3,6-dimethyl-1,4-dioxane-2,5-dione) which is polymerized to high molar mass polymers using ring-opening reactions (101). ROP is the most commonly used method for PLA production, as this chemical process can be controlled and the properties of the polymers varied for different applications (104, 109). ROP is also used to incorporate other ring formed monomers such as glycolide into the lactide polymer. These copolyesters are described later in this review.

Due to the diverse properties of PLA polymers caused by its constituent lactide and the various polymerisation methods, PLA has been processed for a variety of different applications in the biomedical field (110). These include suture lines (111), orthopedic implants (112) and microspheres (3, 113). Among these applications, the microspheres have been reported as being of most interest, because of their unique structure and tailorable properties (114). The remainder of this section will therefore focus on particles made of PLA, their preparation and applications.

PLA particles

PLA-based particles are now widely used in medicine as long-circulating carriers for the controlled release of pharmacological substances (115) such as drugs (116, 117), hormones (118) and for the delivery of proteins and antigens (11, 119, 120). One of the

most important features of particles used for this purpose is the amount of control over the release rate of the desired substance in the recipient tissues. To this end, structural properties of the particles are often tailored and much research has been conducted in this area (98, 116, 121). PLA can be formulated into particles of a wide size range, from nanoparticles and nanocapsules to microspheres and microcapsules. The term microsphere is generally used to describe small, porous spheres from 1-500 μm in diameter, whereas nanoparticles are generally 10-1000 nm in size (2). In drug delivery, particle size is a fundamental parameter which determines the route of administration of the drug (3). Microspheres can be administered intramuscularly or subcutaneously (122) but for intravenous administration and transport via blood circulation or across the mucosal membrane, and in the case of oral administration, particles in the nm range (and particularly less than 500 nm) are required (2, 3, 122). The significance of particle size is basically related to the rate and extent to which the particles are removed from the circulation and their distribution in tissues (123). The size of the particles also affects loading of the drug and its release from the particles into the tissues. Katare *et al.* (11) investigated the size of PLA microparticles in a study of the delivery of the tetanus toxoid (TT) by a single point intramuscular injection in rats. Parameters such as antigen load and the use of extra adjuvant were also considered for eliciting the optimal antibody response. Size ranges for the PLA particles with entrapped antigen were 50-150 μm, 10-70 μm, 2-8 μm and less than 2 μm. They reported that the greatest encapsulation efficiency (60.4%) and TT load (53.2 μg /mg) were obtained with the particle size range of 2-8 μm. Serum antibody titres varied extensively depending on the size of the particles. Once again, particles of 2-8 μm elicited the highest titres whereas in the largest size particle range the titre was considerably lower. Interestingly, particles of less than 2 μm exhibited a low titre. The authors suggested that the differences were related to the uptake of the particles by macrophages, and that since the upper limit for this was considered as 5 μm, the 2-8 μm range was optimal. The lower response of the smallest group of less than 2 μm was thought to be due to less efficient antigen processing.

Apart from size, factors which influence the performance of microparticles include porosity (and the related % solid content) (114, 116), surface charge (124) and hydrophobicity (125). As with size, the optimum degree for porosity will depend on the situation for which they are prepared. For example, microspheres with a highly porous

interior structure are considered as more advantageous than non-porous particles for the administration of inhaled drugs. The large size to density ratio of porous microspheres has been reported to generate a more easily aerosolized particle, resulting in a higher uptake of the inhaled drug (114). On the other hand, for the treatment of arthritic conditions, a less porous particle type has been reported as more suitable by Cui *et al.* (116). This study compared the entrapment and release of the drug melittin, by particles of PLA and the polylactide-glycolide copolymer PLGA. The level of porosity of the two types of polymer particles was different, with the pure PLA particles being more porous and sponge-like, whereas increasing the molar fraction of the glycolide in the PLGA particles resulted in a harder, smoother surface. Although the results showed a significant "initial burst" release in both particle types, it was particularly high for the pure PLA, suggesting a diffusion of the substance through the pores and indicating the lesser efficiency of pure PLA particles in this instance.

The ability to control the rate and extent of the release of the substance is of paramount importance for PLA and other particles used in drug/antigen delivery systems. For this reason, PLA particles are frequently coated with carbohydrates (126, 127), electrolytes (128), polyethylene glycol (129) and other substances to increase stability (130), period of release (128) and blood-circulation time, and to reduce initial burst (131).

Preparation of PLA particles

Several methods are available for the preparation of PLA micro- and nano-particles. It is important to note that the method of preparation and the experimental parameters involved will directly influence the particles produced and their ability to interact with the drugs/antigens to be delivered (3, 114, 132). The three main preparation methods are the (a) emulsion-based solvent evaporation methods, (b) the spray-drying method and (c) nanoprecipitation. Usually, adaptations of the first two methods are used for the microencapsulation of proteins and drugs in PLA particles. Emulsion-based methods consist of four steps: (i) dissolution of the bioactive compound in an organic solvent (ii) formation of an emulsion of the organic solvent phase with an aqueous phase (iii) extraction of the solvent by evaporation which hardens the droplets into solid microparticles and (iv) drying of the microsphere particles (133). The solvent extraction/evaporation method has been used extensively, for example to prepare microspheres coated with collagen (114), enclosed pesticides (113), adsorbed vaccine antigens (134), and encapsulated drugs (135-137) among other medical applications of

PLA particles. In the study of Takei *et al.* (113), this method was used to encapsulate a pesticide acetamiprid into PLA microspheres. The entrapment efficiency of the pesticide decreased with increasing concentration of this chemical in the oil phase. An important part of the process in the solvent emulsion method is the addition of stabilizing agents to the initial droplets and to the final particles. Examples of such surfactants are sodium dodecyl sulfate and cetyltrimethyl ammonium chloride (115), alginate, polyvinylpyrrolidone and gelatin (122, 132). In terms of particle size, PLA nanoparticles and nanospheres are produced by the same methods as microparticles and microspheres, with the manufacturing parameters adjusted to generate nanometer sized droplets, essentially by using a smaller ratio of the droplet phase to the suspension and a much higher stirring rate (138).

The spray-drying method for preparation of PLA particles involves dispersion of the drug in an organic solution of the PLA polymer, followed by immediate nebulization in a hot air flow (3). This method was used by Blanco *et al.* (2006) (15) to prepare microspheres of PLA and other co-polymeric (various ratios of PLA:PLGA) particles. The PLA was dissolved in 2% dichloromethane with constant stirring, then sprayed through the nozzle of a spray-drying device. The microspheres were collected in the separator of the spray-dryer and dried in a vacuum oven. PLA particles produced by this method were small (1.3 µm), slightly porous and with a smooth surface. The particles were analyzed for their degradation rates, and it was apparent that the pure PLA particles showed the slowest rate of degradation. Bishara *et al.* (139) used a similar method from D,L-PLA, producing PLA particles of 2-4 µm. It has been suggested that while this method may be suitable for industrial-scale production of microspheres, the non-uniform particle sizes would be a likely limitation (3).

The nanoprecipitation method for the preparation of PLA particles is a relatively easy method, in which an organic solution of the polymer is simply added to water. PLA particles will immediately form by precipitation, following which the organic solvent can be removed by evaporation (106, 123). Mainly hydrophobic compounds are entrapped by this method (3). Peltonen *et al.* (140) used nanoprecipitation to investigate improved entrapment efficiency of the bronchoconstriction-reducing drug sodium chromoglycate, within PLA nanoparticles. They reported that the loading of the hydrophilic drug into the hydrophobic nanoparticles was difficult but that it was improved by lowering of the pH of the outer medium. Hyvonen *et al.* in their

nanoprecipitation study of PLA nanoparticles reported that the crystallinity of the particles decreased upon nanoprecipitation (141).

Applications of PLA particles

While the physicochemical, chemical and mechanical properties of PLA polymers make them more suitable than other non renewable source based polymers for manufacturing products for industrial packaging, their use in the medical device market as drug delivery and therapeutic devices has greatly appreciated over the past few decades (142-145). In particular, micro and nanospheres, particles and capsules made of PLA on its own or combined with other polymers have received tremendous attention as a means of delivering drugs (1, 4, 146). The advantages of using biodegradable polymers lie in controlling the release of drugs or proteins and their stability. Moreover, their degradation by simple hydrolysis of the ester bond and does not require the presence of enzymes to catalyse this hydrolysis.

Previously, micellar oil-dispersions, liposomes and hydrophilic polymer-photosensitizer conjugates have been used as potential drug carriers with varying degrees of success. PLA and their copolymers have now become the most important polymers for drug delivery systems and were approved by the Food and Drug Administration (147). This is because their biodegradation leads to pharmacologically inactive substances, which are absorbed by the body or removed by the metabolism (148).

Biodegradable PLA makes it an appropriate carrier to deliver drugs which are lipophilic in nature. In ovarian cancer diagnosis, the photodetection (PD) method using photosensitizers (PS) is used to accurately pinpoint the location of malignant tissue and scattered micrometastases in advanced cancer stages (149).While selective accumulation of the drug by tumor to muscle is essential, avoiding the loss of drugs from normal vessels and lowering of PS accumulation in the healthy surrounding tissues is equally important to decrease the side effects such as skin photosensitivity. Also, due to the lipohilic nature of the exogenous PS such as Hypercin (Hy), systemic administration is problematic. This can be mitigated by entrapping PS in biodegradable polymers as carrier materials. Zeisser-Labouebe (150) used PLA nanoparticles loaded with Hy to study the fluorescence PD of ovarian metastases in Fischer 322 rats bearing ovarian tumours. They observed that using PLA nanoparticles resulted in higher selective accumulation and bioavailability of the drug within ovarian micrometastases

when compared to using the free drug, thereby improving PS availability within tumours. In addition, lower concentrations of Hy were found in healthy surrounding tissues and their concentration decreased faster when PLA nanoparticles were used, confirming rapid elimination of Hy from the surrounding healthy tissues.

The selectivity of PS loaded PLA nanoparticles is also favourable for Photo Dynamic Therapy (PDT). PDT is a method in cancer treatment in which the cancer cells and tissues are destroyed by the uptake of PS, followed by photoirradiation (146). PLA nanoparticles would allow targeted delivery because of the possibility to chemically cross-link specific proteins to their surface (117, 151).

In parenteral administration, the PLA nanoparticles are opsonised by mononuclear phagocytes, removed from the blood stream and sequestered in organs such as liver and spleen, avoiding the effective delivery of the nanoparticles to organs other than those of the reticuloendothelial system (152). In a study by Bazile (153) a radiolabelled PLA coated with human serum albumin ($[^{14}C]$-PLA$_{50}$, where 50 refers to the percentage of L-forms of lactic acid within the chains) was synthesized to follow the fate of this new drug carrier after intravenous administration to rats. From whole-body autoradiography and quantitative distribution experiments they observed that the ^{14}C-labelled polymer is rapidly accumulated in liver, bone marrow, lymph nodes, spleen and peritoneal macrophages. To control the opsonization process and to improve the surface properties of the system, the surface of the nanoparticles was modified by either attaching or coating with a hydrophilic polymer such as polyethylene glycol (PEG) (4, 154).

Despite its advantages, the hydrophobic nature of PLA tends to result in adsorption of non specific proteins, which reduces the effectiveness of the drug delivery. This uncontrolled protein adsorption to the lipophilic PLA was combated by synthesizing a diblock copolymer with hydrophilic PEG (155). Also, in oral administration of drugs, there is evidence that PLA nanoparticles are degraded by enzymes in the body fluids such as intestinal fluids and that this degradation is affected by the surface composition of the particles (153). In a study by Landry *et al.* (156), degradation of albumin coated PLA$_{50}$ was performed in simulated gastric and intestinal fluids (USP XXII) in order to model the degradation process. These authors observed that once the readily digestible albumin coat was digested by gastric and intestinal fluids, the exposed PLA$_{50}$ was degraded, mainly due to an enzymatic cleavage process. They concluded that coating PLA with slowly digestible or non-digestible agents would be beneficial in a drug delivery system.

Quantum dots (QD) or semiconductor nanocrystals are nanoparticles with a typical diameter of 2-8 nm that possess unique luminescent properties (157, 158). QDs have been used as novel fluorescent markers in biological labelling and diagnostics (159, 160). Since QD molecules are neither water soluble, biocompatible or have functional groups linked with biomolecules, many of these applications require a combination of the QDs with polymers (157, 161). The polymers are versatile surface modifiers because of their processability and multiple chemical functionalities (162). In a study by Guo *et al.* (163), CdSe (Cadmium and Selenium) QDs were modified to make them water soluble and emit strong fluorescence, by incorporating the CdSe QDs with PLA. The fluorescence of the modified CdSe QDs was stable for more than 30 days *in vitro*. They suggested that the use of PLA could reduce the possibility of QDs loss from the particles. The results demonstrated that PLA particles encapsulated with CdSe QDs had high potential for biological labelling especially in aqueous biological conditions. QDs are known for their cytotoxicity, due to desorption of free Cd (QD core degradation), free radical formation, and interaction of QDs with intracellular components (159). Polyethylene glycosylated (PEG) QDs have been shown to have reduced cytotoxicity, but modification of these to produce PEG-amine for biological activity renders them cytotoxic once again (164). To overcome the problem of cytotoxicity, Guo *et al.* (165) modified CdSe QDs by incorporating these into poly(D, L)-lactide (PDLA) nanoparticles. The resulting luminescent nanoparticles were coated with a layer of different molecules including F-68, cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS). The results suggested that CdSe QDs surface modified with F-68 have low cytotoxicity.

Poly(lactic-co-glycolic acid)

Background

PLGA is a co-polymer which is used in a host of Food and Drug Administration (FDA) approved therapeutic devices, owing to its biodegradability and biocompatibility. Unlike the homopolymers of lactic acid (polylactide) and glycolic acid (polyglycolide) which display poor solubilities, PLGA can be dissolved by a wide range of common solvents, including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate.

PLGA nanoparticles are biodegradable, biocompatible and approved for human use by the FDA. PLGA is hydrolysed to lactic and glycolic acids, both of which are by-

products of various metabolic pathways in the body. By altering the ratio of the monomers used during synthesis, the degradation rate can be controlled (1), making PLGA a common choice in the production of a variety of biomedical devices such as: grafts, sutures, implants, prosthetic devices, micro and nanoparticles.

Preparation of PLGA nanoparticles

While there have been differing techniques employed to produce nanoparticles, the emulsification-diffusion technique was developed in 1996 to overcome toxicity issues with solvents and low yields obtained with gelatine stabilisers. There are single (oil-in-water) and double (water-in-oil-in-water) emulsification techniques (49). Each methodology results in a different release profile of the drug. Propylene carbonate (PC) was added as an alternative low toxicity stable solvent for the polymers (166). In this method, the polymer is dissolved in PC and then emulsified via homogenization with an aqueous surfactant added. The addition of water while stirring allows the PC to diffuse out of the polymer emulsion into the water causing precipitation of the polymer into nanoparticles. The size of the nanoparticles reportedly decreased as the stabiliser concentration increased up to 5% w/v but ultimately the size was determined by the globule size throughout the process of emulsification. The nanoparticle size is therefore influenced by the concentration of the polymer, the rate the emulsification solution is stirred and the concentration of the stabilizer (166).

Various techniques are used to form drug-polymer micro- or nano-particles. A weakness with the use of implants is that the preparative process involves high or elevated temperatures, non uniform content and large variations in individual drug release patterns and of course the surgery required to implant them (167). Biodegradable microparticles developed to overcome this are usually formed by solvent evaporation or organic phase separation, spray drying or supercritical fluid technology, all of which involve several steps within each process and many formulation factors to be controlled (167). Again, rapid release, difficulty with injecting the viscous solution and concerns about the toxicity of the solvents used has led to alternative production methods involving a polymer phase emulsified into an external phase. The partial water miscibility of the organic solvents allowed the properties of the microparticles to be manipulated resulting in porous microparticles due to the polymer solidifying and trapping organic solvents within (167). It was found that by having an external oil

phase, precipitation of the polymer was significantly lower than in implants resulting in reduced initial drug release (167).

The size also affects the distribution of targeted and non-targeted nanoparticles. A study by Cheng *et al.* (168) demonstrated that there were at least four parameters to consider: the concentration of polymer, the drug loading, the water miscibility of the solvent used and the ratio of water to solvent. Likewise, aggregation can be triggered by temperature using pluronics as solvents and particle size can be predicted from the concentration of PLGA used (169).

Differences in formulation can affect the *in vivo* uptake of drugs (8) and similarly polymer degradation is influenced by a number of factors: co-polymer composition, molecular weight and size of particles (1). These are all optimised for the drug used and the therapeutic requirements. In addition to the above applications, modifications and polymer combinations expands the realm of PLGA uses. PLGA can be combined with chitosan to decrease the drug release rate while enhancing particle uptake by cells (170, 171). Cationic drug delivery has been investigated using PLGA based particles incorporating polyvinyl sulphonate-covinyl alcohol-graft-PLGA [P(VS-VA)-g-PLGA] (74). In this study degradation rate was determined by sulphonation.

Surfactants may be used in the formulation of PLGA variants. Polysorbate or poloxamer 188 coated PLGA allows some drugs to be transported across the blood-brain barrier (172). Sodium dodecyl sulphate was used by Xu and Czernuszka (173) while Feczkó *et al.* (174) compared the effect of three different surfactants on the preparation of PLGA. In addition, pluronics, non-ionic macromolecular surface active agents, are used for the solubilisation and controlled release of drugs and have been incorporated into PLGA to produce an amphiphilic co-polymer which enhances protein loading and stability (173).

Application of PLGA in drug delivery

PLGA is a good candidate for therapeutic agent delivery. Drug delivery is often limited by the ability to target drugs to a specific site or tissue. In addition, efficient cytosolic delivery is difficult because the receptors for the drugs are in the cytosol or an organelle must transport the drug to the site. The surface of the nanoparticle and the way it interacts with the cell affects the uptake and subsequent intracellular trafficking of the nanoparticle containing or carrying the therapeutic agent (175). PLGA nanoparticles can be formulated to encapsulate low molecular weight drugs or macromolecules such

as proteins or plasmid DNA (175). The polymer matrix helps to prevent premature degradation of the drug allowing a slower release. Usually around 100 nm in diameter, PLGA nanoparticles are taken up by cells via endocytosis and the material inside the nanoparticle released slowly as the particle degrades. However around 85% of the internalized nanoparticles have been found to undergo exocytosis, i.e. become released again from the cells without release of the drug (176). Adequate uptake by cells is also important if the nanoparticles are to deliver therapeutic agents intracellularly. In the study by Vasir and Labhasetwar (176), PLGA nanoparticles were functionalised with poly-L-lysine (PLL) on the surface and showed a three-fold increase in cellular uptake compared to unmodified PLGA nanoparticles.

PLGA is already used for human drug delivery for a range of medications with FDA approval. A review by Wischke and Schwendeman (177) notes 21 products which have been/are marketed for slow release of compounds. For example, Risperdal Consta® which is used to treat schizophrenia is given 2 weekly while Lupron Depot® may be administered 1-3 monthly for endometriosis or fibroids and every 4 months for prostate cancer treatment. These are all administered by the intra-muscular route while Arestin® microspheres which incorporate an antibiotic (mimocycline) in PLGA beads and are used to treat periodontal disease in adults, are administered into the periodontal socket.

Parenteral administration of drugs involves safety concerns, is inconvenient and tiresome especially for the non-hospitalized person who requires frequent medication. Therefore oral delivery is sought and investigated by many research groups and companies. However, many drugs cannot presently be orally delivered due to difficulties related to degradation in the acidic and protease-rich environment of the gastrointestinal tract. Formulation strategies involving PLGA are being used to investigate oral delivery of many drugs (178), although none have yet been licensed for human use. Some examples showing the range of drugs being investigated are: Doxorubicin, a potent anticancer drug was loaded into orally delivered nanoparticles and results in rats showed enhanced bioavailability and lower toxicity (179); oral delivery of Eudragit® microparticles of PLGA entrapping insulin were evaluated in diabetes induced rats and successfully mediated stability of blood sugar levels (180). The anti-cancer drug 5-fluorouracil was incorporated into PLGA nanoparticles and showed enhanced bioavailability following oral delivery (181).

Topical use of PLGA loaded with flurbiprofen as a treatment for post-ophthalmic surgery pain and inflammation has been investigated *in vitro* and *ex vivo* with promising results (182).

Vaccine delivery has utilised PLGA in different formulations (183). Although effective tuberculosis vaccines remain elusive, studies have used PLGA-based vaccines with some success (184). However, alternative delivery systems were still found to be more effective. Moreover PLGA is also being investigated for oral delivery of vaccines for oral immunotherapy (185), hepatitis B (186) and rotavirus vaccines (187).

Also studied for gene delivery, nanoparticles as a carrier have the gene of interest encapsulated inside a polymer matrix, which could then be used for gene therapy (188). Gene silencing using si(RNA) which incorporates polyethylenimine (PEI) as a cationic polymer into the PLGA matrix has recently undergone evaluation (189). PLGA-coated plasmid DNA has applications in the fields of vaccine delivery and disease treatment. With regard to vaccine delivery, PLGA vaccinations using antigens relevant to human infections have been carried out in mice using different routes of administration (190).

Specific targeting utilising PLGA particles has recently attracted interest by scientists. Magnetic PLGA nanoparticles have been developed and could be directed by externally applied magnetic fields to enable tissue-specific drug delivery (191). Similarly, magnetic targeting has also been applied for cochlear treatment (192).

Delivery of chemotherapy drugs for treatment for retinoblastoma using PLGA nanoparticles has been studied and results indicated that PLGA loaded nanoparticles can provide a degree of sustained delivery (193).

Polymalate

Background

Polymalate, the anion of poly(β-malic acid), is a biopolyester produced by slime moulds and some fungi (194). The polyester backbone is formed by condensation of the hydroxyl groups and the β-carboxyl groups of the linearly arranged malate monomers, while the α-carboxyl groups represent side groups of the polymer backbone (195). This polyester can also be chemically produced from the corresponding lactones (196). PMLA is water-soluble, biodegradable and biocompatible as well as it can be chemically modified to alter its properties. Recently, poly(β-malic acid) and its

derivatives have been investigated with regard to their potential for biomedical applications (197). PMLA is non toxic *in vitro* and *in vivo*, non-immunogenic, stable in the bloodstream and cells can easily take up the polymer (197-201). Polymalate is especially suitable for temporary therapeutic applications (197) and because of its properties more beneficial for repetitive treatment than, for example, viral delivery vectors (202-204).

Natural production of poly(malic acid) was first reported for *Penicillium cyclopium* (205-207) and later also for *Aureobasidium pullulans* (208-210). The best studied producer is the slime mould *Physarum polycephalum* (195, 197, 211). Phylogenetically, *P. polycephalum* belongs to the multicellular eukaryotes (212). During their life cycle slime moulds (myxomycetes) develop a plasmodium that differentiates into spores and later into single-celled amoebae, which might again form a plasmodium after mating (213). The plasmodium is a vegetative multinuclear giant cell with synchronously dividing nuclei. Only in the plasmodium state myxomecetes produce PMLA, it accumulates to high concentrations in the nuclei (214-216).

Although the biochemical pathways are not fully revealed yet, it seems that PMLA functions as a storage and carrier molecule for proteins required to maintain the synchronous division of the nuclei in the giant plasmodium cell (214, 217, 218). Polymalate mimics the distance of the phosphate groups in the DNA backbone (219) and therefore competes with DNA for the binding of histones, DNA polymerases and other nucleic proteins (195, 217-220). Binding to polymalate causes inactivation of the DNA polymerase (195). To maintain the synchronicity of the dividing nuclei a tight regulation between DNA polymerase, histones and other nucleic acid binding proteins is necessary. The level of polymalate remains constantly high in the nuclei and excess polymer is exported into the culture medium and cleaved to L-malate by polymalatase (211).

So far, no PMLA synthase activity has been found in plasmodial lysates but *in vivo* studies indicated the existence of a PMLA synthetase with β-L-malyl-AMP ligase and PMLA polymerase activity. The results suggested that the polymerase activity is regulated by a GTPase-dependent signal pathway which leads to inactivation of the enzyme when the plasmodium is injured (221).

Preparation of Polymalate particles

Polymalate is a water-soluble polymer which can be easily modified at the α-carboxyl groups. Although it can be produced chemically, most applications favour to use the naturally produced optically pure material. In most cases, polymalate is isolated from the natural producer *Physarum polycephalum*. The polymalate producing plasmodia of this slime mould are grown in shaking Erlenmeyer flasks for 2 to 3 days at 21°C to 27°C, depending on the production strain. The actual polymer is isolated from the liquid culture medium using adsorption to DEAE-cellulose as first purification step. Several batches can be combined and then further purified. Pure polymalate is obtained by repeated chromatography on DEAE-cellulose, alcohol precipitation, size exclusion chromatography and lyophilisation (222). It is advantageous to use relatively young cultures for the polymer purification as hydrolytic decomposition to L-malate starts from the onset of the growth. Early stage cultures also assure a polymer with a high-average molecular mass, ranging from 50 kDa to several hundred kDa (194, 222).

Different methods are used to form water insoluble polymalate particles; most of them involve at least one chemical modification step. In the emulsion solvent evaporation method, naturally produced polymalate is modified to a methyl ester which can then be dissolved in chloroform. Depending on the application the drug to be loaded is mixed into the polymer solution and stirred until the solvent evaporated completely. Emulsifiers can be added to facilitate mixing. During the solvent evaporation, water-insoluble microspheres are formed which are rinsed in the end with distilled water and recovered by freeze-drying (223). The same method can be used to mould distinct shapes. Polymalic acid can be mixed with surfactants to form stable ionic complexes which are mixed with the respective application partner and dissolved in chloroform. The mixture can then be poured into moulding forms, e.g. discs, the solvent evaporates and the remaining spheres are available for the designed application (224).

In chromatographic applications, sepharose displaying polymalate is employed to purify polymalate-binding proteins from cell extract. For this purpose the polymer needs to be highly pure. Minimization of covalent binding of malyl residues to the sepharose is necessary and can be achieved using different amines and amides as supplements (225).

The two-step precipitation-dialysis method is used to form nanoparticles of different organic esters of polymalic acid. The polymer is dissolved in a water-miscible solvent; the solution obtained is then slowly stirred into water. The nanoparticles will precipitate

and can be filtered and concentrated using rotary evaporation (226, 227) or dialysis against distilled water using a cellulose membrane (9). Depending on the chemical properties of the product and the desired purity, additional alcohol extraction and washing steps might be necessary, as well as adding stabilisers to the original solution (228).

Applications of Polymalate particles

To date, all studies looking at possible applications of PMLA particles have been aiming at drug delivery, trying to benefit from the excellent biocompatibility and biodegradability of PMLA (which is metabolized via the tricarboxylic acid cycle). As pure PMLA is highly water soluble and rapidly hydrolyzed in an aqueous environment, micro- or nano-particles were in all cases generated from modified PMLA, i.e. from copolymers.

Stochiometric ionic complexes of PMLA and alkyltrimethylammonium surfactants (*n*ATMA·PMLA) were considered to have interesting properties for controlled drug release. These copolymers were found to adopt a well defined supramolecular structure with alternating layers of both components (paraffinic and polyester phase) (229). Discs of 5 mm diameter, either pure or loaded with erythromycin, were used to investigate hydrolytic degradation and antibiotic release (224). Apart from a relatively high degradability of *n*ATMA·PMLA complexes in general (compared to similar complexes made with polyglutamic acid), the length of the surfactant alkyl side chain was shown to influence hydrolysis (by determining hydrophobicity and crystallinity). This should enable precise adjustment of the hydrolytic degradability. The only slight reduction of molecular weight during degradation and the detection of malic acid but not medium size polymer chains as degradation products led to the conclusion that *n*ATMA·PMLA complexes are depleted primarily by surface erosion (instead of bulk degradation) which was considered to be favourable for even drug release. Up to 30 wt% erythromycin could be dispersed in the *n*ATMA·PMLA complex where it was found to localize in the paraffinic subphase. The experimental data further indicated that erythromycin release was due to degradation, not diffusion.

In a similar study, microspheres were generated from methylated PMLA (PMLA-Me) and degradation and drug release compared for different methylation degrees (223). The particle diameter could be adjusted in the range of 1-20 μm. Interestingly, PMLA-Me microspheres appeared to degrade more slowly than films of the same polymer.

Both surface and bulk erosion seemed to contribute to the degradation mechanism. As for nATMA·PMLA complexes (224), erythromycin release from PMLA-Me microspheres was found to be determined by polymer degradation, the role of diffusion being negligible (223).

PMLA-Me nanoparticles prepared from 75% methylated PMLA were investigated as protein delivery carriers (9). This copolyester was insoluble in water, but still quite hydrophilic and readily degradable. Nanoparticles prepared in DMSO displayed the most narrow size distribution of all solvents tested (diameter approx. 100 nm) and a high negative value of zeta-potential which was considered to be advantageous as it allows modification of surface-exposed carboxyl groups and prevents nanoparticle aggregation by electrostatic repulsion. The hydrolytic degradation behaviour was similar to that reported for PMLA-Me microspheres (9, 223). Six proteins with different molecular weights and/or isoelectric points were used to load the nanoparticles by encapsulation (coprecipitation of protein and polymer), physical absorption (incubation of freeze-dried nanoparticles in protein solution) or chemical immobilization (activation of surface carboxyl groups of nanoparticles, followed by covalent binding of protein) (9). The loading efficiency was found to depend on both the protein and the entrapment method, and loading affected nanoparticle size and zeta-potential. Similar to the antibiotics in previous studies (223, 224), protein release took place in a time frame corresponding to the hydrolytic degradation of the nanoparticles (9). Conclusions drawn from a comparison of all protein release profiles included: Independent of the loading method, the rate of protein release decreased with increasing isoelectric point, at a constant pH 7.4, at 37˚C. Release profiles for encapsulated and chemically immobilized proteins were similar and suggested that a certain amount of particle degradation was required for protein release. Release profiles of physically absorbed proteins could be divided into two groups according to their isoelectric point. Basic proteins behaved similar to those loaded by the other two methods, while acidic ones were released faster and more evenly. In addition, the effect of loading and release on protein activity was studied and was also found to depend on the loading method.

Martinez Barbosa and coworkers investigated degradation mechanism and *in vitro* cytotoxicity of nanoparticles prepared from another group of PMLA hydrophobic derivatives, poly(benzyl malate) (PMLABe), poly(hexyl malate) (PMLAHe) and poly(malic acid-*co*-benzyl malate) (PMLAH/Be) (226). Nanoparticles were between 100 and 200 nm in diameter. While particles seemed more prone to aggregation at

acidic pH, the pH was not found to have a major influence on degradation rates. The observed decrease in molecular weight was fastest for PMLAH/Be and slowest for PMLABe particles. NMR studies indicated that degradation of all polymers occurred by random hydrolysis. For the *in vitro* cytotoxicity studies, the different nanoparticles and their degradation products, respectively, were incubated with J774 A1 murine macrophage-like cells and the IC_{50} (concentration required to kill 50% of cells) determined. For all polymers, the IC_{50} decreased with increasing degradation time (and thus increasing amount of degradation products) and with increasing incubation time with cells. PMLAH/Be nanoparticles were reported to display the highest and PMLABe particles the lowest toxicity. The authors concluded that the observed cytotoxicity of PMLABe, PMLAHe and PMLAH/Be nanoparticles is caused by degradation products and not by simple contact of nanoparticles with the cell. They further stated that the overall *in vitro* cytotoxicity was relatively low for all tested nanoparticles, but a direct comparison e.g. to pure PMLA was said not to be feasible due to its high water solubility.

With the aim of developing a sustained release formulation, microspheres of the biodegradable polyester poly-vephyllinemalate (Veph-malate) were generated by cross-linking the bronchial dilator Vephylline with malic acid (228). Particle diameters ranged from 1 to 10 μm (230). In comparison to Vephylline itself Veph-malate was found to be essentially non-toxic (228). In addition, release of the drug from Veph-malate microspheres was slower and more even than from Vephylline hydrogels. Further investigation of the release mechanism revealed that a two-step degradation process initially only led to the formation of oligomers and only later to the release of active Vephylline monomer, resulting in a delayed and prolonged effect of the drug compared to pure Vephylline (231). This two-step release profile was found to be pH dependent (230). Overall, the Veph-malate microspheres were considered to be useful as a prodrug, a term used to describe the carrier-attached chemical prior to its sustained release. The Veph-malate microspheres were also considered to be suitable for parenteral application (228, 231).

Conclusion

Biodegradable micro- and nano-particles have become vital tools for a wide variety of applications in research, biotechnology and medicine (Figure 2). This review has addressed the main groups of biopolyester particles in current use, with respect to their composition, preparation and applications. It is very evident from the ever-increasing range of patents and publications, that this is an area of exceptional growth and development.

One promising approach is the engineering of microbial cells for the manufacture of custom-made beads by hijacking the natural storage granule biogenesis system (6). This in particular allows the one-step production of polymer beads with a protein-based function at high density and functional orientation already attached to its surface. This intracellular production also provides the unique advantage of utilizing a natural environment for the functional attachment of the relevant protein, hence enabling the functional display of difficult to express proteins.
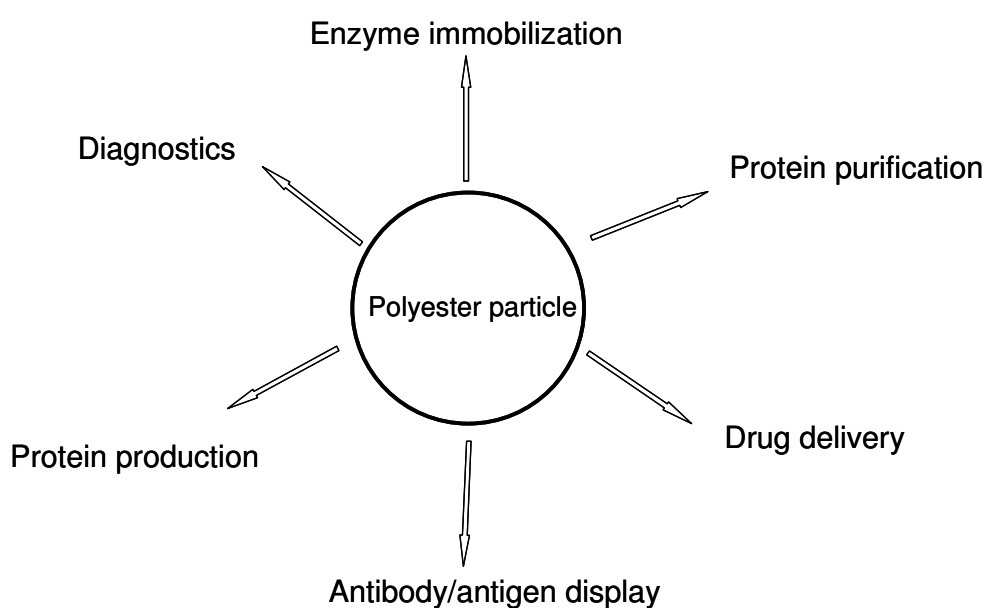

Figure 2: Schematic of biopolyester particle applications

Recently, the use of supercritical fluids (SCF) has gained attention as an advanced method for many biotechnological processes, including the formation of drug encapsulated PLA and PGLA particles (232-234). In addition, SCF has been used to incorporate food materials into biopolyester particles, such as the incorporation of β-

carotenoid into particles of the PHB co-polymer with 3-hydroxyvalerate (PHBV) (235). In these methods, carbon dioxide and/or water in the supercritical state are used to co-precipitate a bioactive substance and the biopolymer, such as in the microencapsulation of the corticosteroid budesonide into PLA particles (136, 236). The use of carbon dioxide in the supercritical state is reported as being useful for producing particles in a more controlled fashion than the conventional methods (232) and to enhance the solubility of drugs which are poorly soluble in aqueous and organic media (234).

While the use of biopolyester particles continues to grow in many areas, there are several challenges that remain to be overcome. For example, it seems that the size and uniformity of the particles have been identified as particularly important parameters in many contexts. In addition, the reduction of initial burst and solubility improvement are critical issues in drug delivery. In industrial settings, product quality is often dependent on the level of existing control over the physical characteristics of the particulate vehicles. Similarly, medical applications require particle characteristics that promote consistency and reproducibility. Therefore, much effort is now being invested in improving and tailoring particle properties, for bacterial as well as synthetically produced biopolymer particles. There is no doubt that the development and fine-tuning of these particles, and their potential applications, will continue to expand well into the future.

References

1. J.M. Anderson and M.S. Shive, Adv. Drug Del. Rev., 1997. **28**: p. 5-24.
2. F. Mohamed and C.F. van der Walle, J. Pharm. Sci., 2008. **97**(1): p. 71-87.
3. V. Lassalle and M.L. Ferreira, Macromol. Biosci., 2007. **7**(6): p. 767-783.
4. K.S. Soppimath, T.M. Aminabhavi, A.R. Kulkarni, and W.E. Rudzinski, J. Controlled Release, 2001. **70**(1-2): p. 1-20.
5. T.B. Bäckström, J.A. Brockelbank, and B.H.A. Rehm, BMC Biotechnol., 2007. **7**(1): p. 3.
6. B.H. Rehm, Curr. Issues Mol. Biol., 2007. **9**(1): p. 41-62.
7. D.M. Horowitz and J.K.M. Sanders, J. Am. Chem. Soc., 1994. **116**(7): p. 2695-2702.

8. R.C. Mundargi, V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, J. Controlled Release, 2008. **125**(3): p. 193-209.

9. J.A. Portilla-Arias, M. Garcia-Alvarez, J.A. Galbis, and S. Munoz-Guerra, Macromol. Biosci., 2008b. **8**(6): p. 551-559.

10. S. Gelperina, K. Kisich, M.D. Iseman, and L. Heifets, Am. J. Respir. Crit. Care Med., 2005. **172**(12): p. 1487-1490.

11. Y.K. Katare, T. Muthukumaran, and A.K. Panda, Int. J. Pharm., 2005. **301**(1-2): p. 149-160.

12. M. Peyre, R. Audran, F. Estevez, G. Corradin, B. Gander, D. Sesardic, and P. Johansen, J. Controlled Release, 2004. **99**(3): p. 345-355.

13. E. Catiker, M. Gumusderelioglu, and A. Guner, Polym. Int., 2000. **49**(7): p. 728-734.

14. B.S. Lee, M. Fujita, N.M. Khazenzon, K.A. Wawrowsky, S. Wachsmann-Hogiu, D.L. Farkas, K.L. Black, J.Y. Ljubimova, and E. Holler, Bioconj. Chem., 2006. **17**(2): p. 317-326.

15. M.D. Blanco, R.L. Sastre, C. Teijon, R. Olmo, and J.M. Teijon, Int. J. Pharm., 2006. **326**(1-2): p. 139-147.

16. M. Dunne, I. Corrigan, and Z. Ramtoola, Biomaterials, 2000. **21**(16): p. 1659-1668.

17. F. Alexis, Polym. Int., 2005. **54**(1): p. 36-46.

18. G. Schwach, J. Coudane, R. Engel, and M. Vert, Biomaterials, 2002. **23**(4): p. 993-1002.

19. M.R. Banki and D.W. Wood, Microb. Cell Fact., 2005. **4**: p. 32.

20. O. Salata, J. Nanobiotechnology, 2004. **2**(1): p. 3.

21. V. Peters and B.H.A. Rehm, J. Biotechnol., 2008. **134**: p. 266-274.

22. A.C. Jahns, R.G. Haverkamp, and B.H.A. Rehm, Bioconjug. Chem., 2008. **19**(10): p. 2072-2080.

23. K. Grage and B.H.A. Rehm, Bioconjug. Chem., 2008. **19**(1): p. 254-262.

24. I.A. Rasiah and B.H. A. Rehm, Appl. and Environ. Microbiol., 2009. **75**(7): p. 2012-2016.

25. A.J. Anderson and E.A. Dawes, Microbiol. Rev., 1990. **54**(4): p. 450-472.

26. R. Fernandez-Castillo, F. Rodriguez-Valera, J. Gonzalez-Ramos, and F. Ruiz-Berraquero, Appl. Environ. Microbiol., 1986. **51**(1): p. 214-216.

27. J. Han, Q. Lu, L. Zhou, J. Zhou, and H. Xiang, Appl. Environ. Microbiol., 2007. **73**(19): p. 6058-6065.

28. F.F. Hezayen, B.H.A. Rehm, R. Eberhardt, and A. Steinbüchel, Appl. Microbiol. Biotechnol., 2000. **54**(3): p. 319-325.

29. L.L. Madison and G.W. Huisman, Microbiol. Mol. Biol. Rev., 1999. **63**(1): p. 21-53.

30. S.P. Valappil, A.R. Boccaccini, C. Bucke, and I. Roy, Antonie Van Leeuwenhoek, 2007. **91**(1): p. 1-17.

31. G. Braunegg, G. Lefebvre, and K.F. Genser, J. Biotechnol., 1998. **65**(2-3): p. 127-161.

32. G.A. van der Walle, G.J. de Koning, R.A. Weusthuis, and G. Eggink, Adv. Biochem. Eng./Biotechnol., 2001. **71**: p. 263-291.

33. P. Furrer, S. Panke, and M. Zinn, J. Microbiol. Methods, 2007. **69**: p. 206-213.

34. B. Hazer and A. Steinbüchel, Appl. Microbiol. Biotechnol., 2007. **74**(1): p. 1-12.

35. O.P. Peoples and A.J. Sinskey, J. Biol. Chem., 1989. **264**(26): p. 15298-15303.

36. O.P. Peoples and A.J. Sinskey, J. Biol. Chem., 1989. **264**(26): p. 15293-15297.

37. A. Steinbüchel and H.G. Schlegel, Mol. Microbiol., 1991. **5**(3): p. 535-542.

38. R. Griebel, Z. Smith, and J.M. Merrick, Biochemistry (Mosc). 1968. **7**(10): p. 3676-3681.

39. M. Liebergesell, K. Sonomoto, M. Madkour, F. Mayer, and A. Steinbüchel, Eur. J. Biochem., 1994. **226**(1): p. 71-80.

40. D. Gao, A. Maehara, T. Yamane, and S. Ueda, FEMS Microbiol. Lett., 2001. **196**(2): p. 159-164.

41. R. Handrick, S. Reinhardt, and D. Jendrossek, J. Bacteriol., 2000. **182**(20): p. 5916-5918.

42. S.Z. Hanley, D.J. Pappin, D. Rahman, A.J. White, K.M. Elborough, and A.R. Slabas, FEBS Lett., 1999. **447**(1): p. 99-105.

43. D. Jendrossek and R. Handrick, Annu. Rev. Microbiol., 2002. **56**: p. 403-432.

44. A. Maehara, S. Taguchi, T. Nishiyama, T. Yamane, and Y. Doi, J. Bacteriol., 2002. **184**(14): p. 3992-4002.

45. U. Pieper-Fürst, M.H. Madkour, F. Mayer, and A. Steinbüchel, J. Bacteriol., 1995. **177**(9): p. 2513-2523.

46. M. Pötter, M.H. Madkour, F. Mayer, and A. Steinbüchel, Microbiology, 2002. **148**: p. 2413-2426.

47. M. Pötter and A. Steinbüchel, Biomacromolecules, 2005. **6**(2): p. 552-560.

48. M.A. Prieto, B. Buhler, K. Jung, B. Witholt, and B. Kessler, J. Bacteriol., 1999. **181**(3): p. 858-868.

49. R. Wieczorek, A. Pries, A. Steinbüchel, and F. Mayer, J. Bacteriol., 1995. **177**(9): p. 2425-2435.

50. G.M. York, J. Stubbe, and A.J. Sinskey, J. Bacteriol., 2002. **184**(1): p. 59-66.

51. S.Y. Lee, Nat. Biotechnol., 1997. **15**(1): p. 17-18.

52. S.Y. Lee and H.N. Chang, Can. J. Microbiol., 1995. **41 Suppl 1**: p. 207-215.

53. S.Y. Lee, H.N. Chang, and Y.K. Chang, Ann. N. Y. Acad. Sci., 1994a. **721**: p. 43-53.

54. S.Y. Lee, K.S. Yim, H.N. Chang, and Y.K. Chang, J. Biotechnol., 1994b. **32**(2): p. 203-211.

55. Y. Poirier, Adv. Biochem. Eng. Biotechnol., 2001. **71**: p. 209-240.

56. P. Suriyamongkol, R. Weselake, S. Narine, M. Moloney, and S. Shah, Biotechnol. Adv., 2007. **25**(2): p. 148-175.

57. K. Grage, A.C. Jahns, N. Parlane, R. Palanisamy, I.A. Rasiah, J.A. Atwood, and B.H.A. Rehm, Biomacromolecules, 2009. **10**(4): p. 660-669.

58. B. Kessler, R. Weusthuis, B. Witholt, and G. Eggink, Adv. Biochem. Eng./Biotechnol., 2001. **71**: p. 159-182.

59. S.Y. Lee, Biotechnol. Bioeng., 1996. **49**(1): p. 1-14.

60. M. Zinn, B. Witholt, and T. Egli, Adv. Drug Del. Rev., 2001. **53**(1): p. 5-21.

61. H. Brandl, R.A. Gross, R.W. Lenz, and R.C. Fuller, Appl. Environ. Microbiol., 1988. **54**(8): p. 1977-1982.

62. R.G. Lageveen, G.W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt, Appl. Environ. Microbiol., 1988. **54**(12): p. 2924-2932.

63. J. Yao, G. Zhang, Q. Wu, G.Q. Chen, and R. Zhang, Antonie Van Leeuwenhoek, 1999. **75**(4): p. 345-349.

64. E. Berger, B.A. Ramsay, J.A. Ramsay, C. Chavarie, and G. Braunegg, Biotechnol. Tech., 1989. **3**(4): p. 227-232.

65. J.A. Ramsay, E. Berger, B.A. Ramsay, and C. Chavarie, Biotechnol. Tech., 1990. **4**(4): p. 221-226.

66. S.K. Hahn, Y.K. Chang, B.S. Kim, and H.N. Chang, Biotechnol. Bioeng., 1994. **44**(2): p. 256-261.

67. S.K. Hahn, Y.K. Chang, B.S. Kim, K.M. Lee, and H.N. Chang, Biotechnol. Tech., 1993. **7**(3): p. 209-212.

68. J.A. Ramsay, E. Berger, R. Voyer, C. Chavarie, and B.A. Ramsay, Biotechnol. Tech., 1994. **8**(8): p. 589-594.

69. D. Byrom, Trends Biotechnol., 1987. **5**(9): p. 246-250.

70. G.J.M. de Koning, M. Kellerhals, C. van Meurs, and B. Witholt, Bioprocess Eng., 1997a. **17**(1): p. 15-21.

71. G.J.M. de Koning and B. Witholt, Bioprocess Eng., 1997b. **17**(1): p. 7-13.

72. S.F. Williams, D.P. Martin, D.M. Horowitz, and O.P. Peoples, Int. J. Biol. Macromol., 1999. **25**(1-3): p. 111-121.

73. S.J. Lee, J.P. Park, T.J. Park, S.Y. Lee, S. Lee, and J.K. Park, Anal. Chem., 2005. **77**(17): p. 5755-5759.

74. X. Wang, X. Xie, C. Cai, E. Rytting, T. Steele, and T. Kissel, Macromolecules, 2008. **41**(8): p. 2791-2799.

75. Y.-C. Yao, X.-Y. Zhan, J. Zhang, X.-H. Zou, Z.-H. Wang, Y.-C. Xiong, J. Chen, and G.-Q. Chen, Biomaterials, 2008. **29**(36): p. 4823-4830.

76. N. Gangrade and J.C. Price, J. Microencaps., 1991. **8**(2): p. 185-202.

77. G. Khang, S.W. Kim, J.C. Cho, J.M. Rhee, S.C. Yoon, and H.B. Lee, Bio-Med. Mater. Eng., 2001. **11**(2): p. 89-103.

78. J. Chen and S.S. Davis, J. Microencaps., 2002. **19**(2): p. 191-201.

79. J.K. Embleton and B.J. Tighe, J. Microencaps., 2002. **19**(6): p. 737-752.

80. I. Gürsel and V. Hasirci, J. Microencaps., 1995. **12**(2): p. 185-193.

81. P.A. Coimbra, H.C. De Sousa, and M.H. Gil, J. Microencaps., 2008. **25**(3): p. 170-178.

82. J.M. Merrick and M. Doudoroff, J. Bacteriol., 1964. **88**: p. 60-71.

83. G.A. Ritchie and E.A. Dawes, Biochem. J., 1969. **112**(5): p. 803-805.

84. V. Peters and B.H.A. Rehm, Appl. Environ. Microbiol., 2006. **72**(3): p. 1777-1783.

85. T. Fukui, A. Yoshimoto, M. Matsumoto, S. Hosokawa, and T. Saito, Arch. Microbiol., 1976. **110**(23): p. 149-156.

86. M.N. Kraak, T.H. Smits, B. Kessler, and B. Witholt, J. Bacteriol., 1997. **179**(16): p. 4985-4991.

87. M. Liebergesell, B. Schmidt, and A. Steinbüchel, FEMS Microbiol. Lett., 1992. **99**(2-3): p. 227-232.

88. R.G. Martin and B.N. Ames, J. Biol. Chem., 1961. **236**: p. 1372-1379.

89. M.R. Banki, T.U. Gerngross, and D.W. Wood, Protein Sci., 2005. **14**(6): p. 1387-1395.

90. G.C. Barnard, J.D. McCool, D.W. Wood, and T.U. Gerngross, Appl. Environ. Microbiol., 2005. **71**(10): p. 5735-5742.

91. C. Moldes, P. Garcia, J.L. Garcia, and M.A. Prieto, Appl. Environ. Microbiol., 2004. **70**(6): p. 3205-3212.

92. Z. Wang, H. Wu, J. Chen, J. Zhang, Y. Yao, and G.-Q. Chen, Lab. Chip, 2008. **8**(11): p. 1957-1962.

93. J.A. Brockelbank, V. Peters, and B.H. Rehm, Appl. Environ. Microbiol., 2006. **72**(11): p. 7394-7397.

94. J.A. Atwood and B.H.A. Rehm, Biotechnol. Lett., 2009. **31**(1): p. 131-137.

95. T.W. Atkins and S.J. Peacock, J. Microencaps., 1995. **13**: p. 709-717.

96. N. Durán, M.A. Alvarenga, E.C. Da Silva, P.S. Melo, and P.D. Marcato, Arch. Pharmacal Res., 2008. **31**(11): p. 1509-1516.

97. J.K. Embleton and B.J. Tighe, J. Microencaps., 1992. **9**(1): p. 73-87.

98. E. Leo, B. Ruozi, G. Tosi, and M.A. Vandelli, Int. J. Pharm., 2006. **323**(1-2): p. 131-138.

99. R. Auras, B. Harte, and S. Selke, Macromol. Biosci., 2004. **4**(9): p. 835-864.

100. E. Rudnik, *Properties and Applications*, in *Compostable Polymer Materials*. 2008, Elsivier Ltd, Amsterdam, The Netherlands. p. 39-69.

101. A. Södergård and M. Stolt, Prog. Polym. Sci., 2002. **27**(6): p. 1123-1163.

102. Y. Tokiwa and B.P. Calabia, Appl. Microbiol. Biotechnol., 2006. **72**(2): p. 244-251.

103. M. Vert, *Poly(lactide)s*, in *Encyclopedia of Biomaterials and Biomedical Engineering*, G. Wnek and G. Bowlin, Editors. 2004, Marcel Dekker Inc. NY 10016, USA. p. 1254-1263.

104. H. Tsuji, Macromol. Biosci., 2005. **5**: p. 569-597.

105. R. Bhardwaj and A.K. Mohanty, J. Biobased Mater. Bioenergy, 2007. **1**(2): p. 191-209.

106. U. Bilati, E. Allemann, and E. Doelker, Eur. J. Pharm. Sci., 2005. **24**(1): p. 67-75.

107. S.H. Hyon, K. Jamshidi, and Y. Ikada, Biomaterials, 1997. **18**(22): p. 1503-1508.

108. Y. Zhao, Z. Wang, J. Wang, H. Mai, B. Yan, and F. Yang, J. Appl. Polym. Sci., 2003. **91**(4): p. 2143-2150.

109. Y. He, Y. Xu, J. Wei, Z.Y. Fan, and S.M. Li, Polymer, 2008. **49**(26): p. 5670-5675.

110. H. Sawalha, K. Schroen, and R. Boom, J. Appl. Polym. Sci., 2008. **107**(1): p. 82-93.

111. C.-W. Lou, Text. Res. J., 2008. **78**(11): p. 958-965.

112. C.S. Leiggener, R. Curtis, A.A. Muller, D. Pfluger, S. Gogolewski, and B.A. Rahn, Biomaterials, 2006. **27**(2): p. 202-207.

113. T. Takei, M. Yoshida, Y. Hatate, K. Shiomori, and S. Kiyoyama, Polym. Bull., 2008. **61**(3): p. 391-397.

114. Y. Hong, C. Gao, Y. Xie, Y. Gong, and J. Shen, Biomaterials, 2005. **26**(32): p. 6305-6313.

115. A. Musyanovych, J. Schmitz-Wienke, V. Mailander, P. Walther, and K. Landfester, Macromol. Biosci., 2008. **8**(2): p. 127-139.

116. F. Cui, D. Cun, A. Tao, M. Yang, K. Shi, M. Zhao, and Y. Guan, J. Controlled Release, 2005. **107**(2): p. 310-319.

117. J.-C. Olivier, NeuroRX, 2005. **2**(1): p. 108-119.

118. M. Dasaratha D, K. Vema, R. Jayakumar, and C. Vamsadhara, Int. J. Pharm., 2003. **268**(1-2): p. 23-29.

119. A. Vila, A. Sanchez, M. Tobio, P. Calvo, and M.J. Alonso, J. Controlled Release, 2002. **78**(1-3): p. 15-24.

120. J. Wang, B.M. Wang, and S.P. Schwendeman, J. Controlled Release, 2002. **82**(2-3): p. 289-307.

121. V.C.F. Mosqueira, P. Legrand, H. Pinto-Alphandary, F. Puisieux, and G. Barratt, J. Pharm. Sci., 2000. **89**(5): p. 614-626.

122. M. Jobmann and G. Rafler, Int. J. Pharm., 2002. **242**(1-2): p. 213-217.

123. P. Legrand, S. Lesieur, A. Bochot, R. Gref, W. Raatjes, G. Barratt, and C. Vauthier, Int. J. Pharm., 2007. **344**(1-2): p. 33-43.

124. H. Arimura, Y. Ohya, T. Ouchi, and H. Yamada, J. Colloid Interface Sci., 2004. **270**(2): p. 299-303.

125. D.L. Biggs, C.S. Lengsfeld, B.M. Hybertson, K.Y. Ng, M.C. Manning, and T.W. Randolph, J. Controlled Release, 2003. **92**(1-2): p. 147-161.

126. W. Ma, X. Yuan, C. Kang, T. Su, X. Yuan, P. Pu, and J. Sheng, Carbohydr. Polym., 2008. **72**: p. 75-81.

127. C. Nouvel, J. Raynaud, E. Marie, E. Dellacherie, J.L. Six, and A. Durand, J. Colloid Interface Sci., 2009. **330**(2): p. 337-343.

128. S. Hirsjarvi, L. Peltonen, and J. Hirvonen, Colloids Surf .B 2006. **49**(1): p. 93-99.

129. X. Gao, W. Tao, W. Lu, Q. Zhang, Y. Zhang, X. Jiang, and S. Fu, Biomaterials, 2006. **27**(18): p. 3482-3490.

130. M.K. Yeh, J. Microencapsul., 2000. **17**(6): p. 743-756.

131. S. Nishino, A. Kishida, and H. Yoshizawa, Int. J. Pharm., 2007. **330**(1-2): p. 32-36.

132. T.W. Chung, Y.Y. Huang, and Y.Z. Liu, Int. J. Pharm., 2001. **212**(2): p. 161-169.

133. S. Freitas, H.P. Merkle, and B. Gander, J. Controlled Release, 2005. **102**(2): p. 313-332.

134. N. Venkataprasad, A.G. Coombes, M. Singh, M. Rohde, K. Wilkinson, F. Hudecz, S.S. Davis, and H.M. Vordermeier, Vaccine, 1999. **17**(15-16): p. 1814-1819.

135. R. Dinarvand, S.H. Moghadam, L. Mohammadyari-Fard, and F. Atyabi, AAPS PharmSciTech, 2003. **4**(3): p. E34.

136. T.M. Martin, N. Bandi, R. Shulz, C.B. Roberts, and U.B. Kompella, AAPS PharmSciTech, 2002. **3**(3): p. E18.

137. H. Yoshizawa, S. Nishino, K. Shiomori, S. Natsugoe, T. Aiko, and Y. Kitamura, Int. J. Pharm., 2005. **296**(1-2): p. 112-116.

138. R. Arshady, J. Controlled Release, 1991. **17**: p. 1-22.

139. A. Bishara and A.J. Domb, J. Controlled Release, 2005. **107**(3): p. 474-483.

140. L. Peltonen, J. Aitta, S. Hyvonen, M. Karjalainen, and J. Hirvonen, AAPS PharmSciTech, 2004. **5**(1): p. E16.

141. S. Hyvonen, L. Peltonen, M. Karjalainen, and J. Hirvonen, Int. J. Pharm., 2005. **295**(1-2): p. 269-281.

142. R.R.M. Bos, F.R. Rozema, G. Boering, A.J. Nijenhuis, A.J. Pennings, and H.W.B. Jansen, Br. J. Oral Maxillofac. Surg., 1989. **27**(6): p. 467-476.

143. R. Datta, S.-P. Tsai, P. Bonsignore, S.-H. Moon, and J.R. Frank, FEMS Microbiol. Rev., 1995. **16**(2-3): p. 221-231.

144. D. Garlotta, J. Polym. Environ., 2001. **9**(2): p. 63-84.

145. M. van der Elst, A.R.A. Dijkema, C.P.A.T. Klein, P. Patka, and H.J.T.M. Haarman, Biomaterials, 1995. **16**(2): p. 103-106.

146. D.K. Chatterjee, L.S. Fong, and Y. Zhang, Adv. Drug Delivery Rev., 2008. **60**(15): p. 1627-1637.

147. R.E. Conn, J.J. Kolstad, J.F. Borzelleca, D.S. Dixler, L.J. Filer Jr, B.N. Ladu Jr, and M.W. Pariza, Food Chem. Toxicol., 1995. **33**(4): p. 273-283.

148. C. Contado, A. Dalpiaz, E. Leo, M. Zborowski, and P.S. Williams, J. Chromatogr., 2007. **1157**(1-2): p. 321-335.

149. M. Stringer and K. Moghissi, Photodia. Photodyn. Therapy, 2004. **1**(1): p. 9-12.

150. M. Zeisser-Labouèbe, F. Delie, R. Gurny, and N. Lange, Eur. J. Pharm. Biopharm., 2009. **71**(2): p. 207-213.

151. L. Nobs, F. Buchegger, R. Gurny, and E. Allémann, Int. J. Pharm., 2003. **250**(2): p. 327-337.

152. N. Kumar, M.N.V. Ravikumar, and A.J. Domb, Adv. Drug Del. Rev., 2001. **53**(1): p. 23-44.

153. D.V. Bazile, C. Ropert, P. Huve, T. Verrecchia, M. Mariard, A. Frydman, M. Veillard, and G. Spenlehauer, Biomaterials, 1992. **13**(15): p. 1093-1102.

154. B. Pegaz, E. Debefve, F. Borle, J.-P. Ballini, H. van den Bergh, and Y.N. Kouakou-Konan, J. Photochem. Photobiol. B: Biol., 2005. **80**(1): p. 19-27.

155. M. Tobío, A. Sánchez, A. Vila, I. Soriano, C. Evora, J.L. Vila-Jato, and M.J. Alonso, Colloids Surf., B 2000. **18**(3-4): p. 315-323.

156. F.B. Landry, D.V. Bazile, G. Spenlehauer, M. Veillard, and J. Kreuter, Biomaterials, 1996. **17**(7): p. 715-723.

157. W.C.W. Chan, D.J. Maxwell, X. Gao, R.E. Bailey, M. Han, and S. Nie, Curr. Opin. Biotechnol., 2002. **13**(1): p. 40-46.

158. I.G. Min Zhou, Peptide Science, 2007. **88**(3): p. 325-339.

159. T. Jamieson, R. Bakhshi, D. Petrova, R. Pocock, M. Imani, and A.M. Seifalian, Biomaterials, 2007. **28**(31): p. 4717-4732.

160. J. Pan, Y. Wang, and S.-S. Feng, Biotechnol. Bioeng., 2008. **101**(3): p. 622-633.

161. N. Tomczak, D. Janczewski, M. Han, and G.J. Vancso, Prog. Polym. Sci., 2009. In Press.

162. X.-S. Wang, T.E. Dykstra, M.R. Salvador, I. Manners, G.D. Scholes, and M.A. Winnik, J. Am. Chem. Soc., 2004. **126**(25): p. 7784-7785.

163. G. Guo, W. Liu, J. Liang, H. Xu, Z. He, and X. Yang, Mater. Lett., 2006. **60**(21-22): p. 2565-2568.

164. J.P. Ryman-Rasmussen, J.E. Riviere, and N.A. Monteiro-Riviere, J. Invest. Dermatol., 2006. **127**(1): p. 143-153.

165. G. Guo, W. Liu, J. Liang, Z. He, H. Xu, and X. Yang, Mater. Lett., 2007. **61**(8-9): p. 1641-1644.

166. D. Quntanar-Guerrero, H. Fessi, E. Allmann, and E. Doelker, Int. J. Pharm., 1996(143): p. 133-141.

167. H. Kranz and R. Bodmeier, Eur. J. Pharm. Sci., 2008(34): p. 164-172.

168. J. Cheng, B.A. Teply, I. Sherifi, J. Sung, G. Luther, F.X. Gu, E. Levy-Nissenbaum, A.F. Radovic-Moreno, R. Langer, and O.C. Farokhzad, Biomaterials, 2007(28): p. 869-876.

169. M. Fraylich, W. Wang, K. Shakesheff, C. Alexander, and B. Saunders, Langmuir, 2008(24): p. 7761-7768

170. C. Guo and R.A. Gemeinhart, Eur. J. Pharm. Biopharm., 2008. **70**(2): p. 597-604.

171. B.S. Kim, C.S. Kim, and K.M. Lee, Arch. Pharmacal Res., 2008. **31**(8): p. 1050-1054.

172. J. Kreuter and S. Gelperina, Tumori, 2008. **94**(2): p. 271-277.

173. Q. Xu and J.T. Czernuszka, J. Controlled Release, 2008. **127**(2): p. 146-153.

174. T. Feczkó, J. Tóth, and J. Gyenis, Colloids Surf. Physicochem. Eng. Aspects, 2008. **319**(1-3): p. 188-195.

175. J.K. Vasir and V. Labhasetwar, Adv. Drug Del. Rev., 2007. **59**(8): p. 718-728.

176. J.J. Vasir and V. Labhasetwar, Biomaterials, 2008(29): p. 4244-4252.

177. C. Wischke and S.P. Schwendeman, Int. J. Pharm., 2008. **364**(2): p. 298-327.

178. A. des Rieux, V. Fievez, M. Garinot, Y.-J. Schneider, and V. Preat, J. Controlled Release, 2006. **116**(1): p. 1-27.

179. D.R. Kalaria, G. Sharma, V. Beniwal, and M.N.V. Ravi Kumar, Pharm. Res., 2008: p. 1-10.

180. P. Naha, V. Kanchan, P.K. Manna, and A.K. Panda, J. Microencaps., 2008. **25**(4): p. 248-256.

181. X. Li, Y. Xu, G. Chen, P. Wei, and Q. Ping, Drug Dev. Ind. Pharm., 2008. **34**(1): p. 107-115.

182. E. Vega, F. Gamisans, M.L. GarcÃ-a, A. Chauvet, F. Lacoulonche, and M.A. Egea, J. Pharm. Sci., 2008. **97**(12): p. 5306-5317.

183. M. Singh, J. Kazzaz, M. Ugozzoli, P. Malyala, J. Chesko, and D.T. O'Hagan, Curr. Drug Del., 2006. **3**(1): p. 115-120.

184. D.J. Kirby, I. Rosenkrands, E.M. Agger, P. Andersen, A.G.A. Coombes, and Y. Perrie, J. Drug Targeting, 2008. **16**(4): p. 282 - 293.

185. F. Roth-Walter and E. Jensen-Jarolim, Allergy Clin. Immunol. Int., 2007. **19**(1): p. 21-26.

186. P.N. Gupta, K. Khatri, A.K. Goyal, N. Mishra, and S.P. Vyas, J. Drug Targeting, 2007. **15**(10): p. 701-713.

187. B. Nayak, A.K. Panda, P. Ray, and A.R. Ray, J. Microencaps., 2009. **26**(2): p. 154-165.

188. J. Luten, C.F. van Nostrum, S.C. De Smedt, and W.E. Hennink, J. Controlled Release, 2008. **126**(2): p. 97-110.

189. Y. Patil and J. Panyam, Int. J. Pharm., 2009. **367**(1-2): p. 195-203.

190. J. Wendorf, J. Chesko, J. Kazzaz, M. Ugozzoli, M. Vajdy, D. O'Hagan, and M. Singh, Human Vaccines, 2008. **4**(1): p. 44-49.

191. T.K. Jain, J. Richey, M. Strand, D.L. Leslie-Pelecky, C.A. Flask, and V. Labhasetwar, Biomaterials, 2008. **29**(29): p. 4012-4021.

192. K.J. Dormer, V. Awasthi, W. Galbraith, R.D. Kopke, K. Chen, and R. Wassel, J. of Biomed. Nanotechnol., 2008. **4**(2): p. 174-184.

193. E.S. Kim, C. Durairaj, R.S. Kadam, S.J. KLee, Y. Mo, D.H. Geroski, U.B. Kompella, and H.F. Edelhauser, Pharm. Res., 2009.

194. K. Rathberger, H. Reisner, B. Willibald, H.P. Molitoris, and E. Holler, Mycol. Res., 1999. **103**: p. 513-520.

195. H. Fischer, S. Erdmann, and E. Holler, Biochemistry (Mosc). 1989. **28**(12): p. 5219-5226.

196. M. Vert and R.W. Lenz, ACS Polymer Preprints, 1979. **20**: p. 608-611.

197. B.S. Lee, M. Vert, and E. Holler, *Water-soluble aliphatic polyesters: poly(malic acid)s*, in *Biopolymers*, Y. Doi and A. Steinbüchel, Editors. 2002, Wiley-VCH: Weinheim. p. 75-103.

198. C. Braud, C. Bunel, and M. Vert, Polym. Bull., 1985. **13**(4): p. 293-299.

199. S. Cammas, M.M. Bear, L. Moine, R. Escalup, G. Ponchel, K. Kataoka, and P. Guerin, Int. J. Biol. Macromol., 1999. **25**(1-3): p. 273-282.

200. D. Domurado, P. Fournie, C. Braud, M. Vert, P. Guerin, and F. Simonnet, J. Bioact. Compat. Polym., 2003. **18**: p. 23-32.

201. B. Gasslmaier, C.M. Krell, D. Seebach, and E. Holler, Eur. J. Biochem., 2000. **267**(16): p. 5101-5105.

202. A.V. Kabanov, E.V. Batrakova, S. Sriadibhatla, Z. Yang, D.L. Kelly, and V.Y. Alakov, J. Controlled Release, 2005. **101**(1-3): p. 259-271.

203. R. Satchi-Fainaro, M. Puder, J.W. Davies, H.T. Tran, D.A. Sampson, A.K. Greene, G. Corfas, and J. Folkman, Nat. Med., 2004. **10**(3): p. 255-261.

204. S.V. Vinogradov, E.V. Batrakova, S. Li, and A.V. Kabanov, J. Drug Target., 2004. **12**(8): p. 517-526.

205. K. Shimada and Matsushi.K, Agric. Biol. Chem., 1969. **33**(4): p. 544-&.

206. K. Shimada and K. Matsushima, Nippon Nogeikagaku Kaishi, 1967. **41**: p. 454-458.

207. K. Shimada, K. Matsushima, J. Fukumoto, and T. Yamamoto, Biochem. Biophys. Res. Commun., 1969. **35**(5): p. 619-624.

208. S. Liu and A. Steinbüchel, Appl. Microbiol. Biotechnol., 1996. **46**(3): p. 273-278.

209. S.J. Liu and A. Steinbüchel, Biotechnol. Lett., 1997. **19**(1): p. 11-14.

210. N. Nagata, T. Nakahara, and T. Tabuchi, Biosci., Biotechnol., Biochem., 1993. **57**(4): p. 638-642.

211. C. Korherr, M. Roth, and E. Holler, Can. J. Microbiol., 1995. **41**: p. 192-199.

212. S.L. Baldauf and W.F. Doolittle, Proc. Natl. Acad. Sci. U. S. A., 1997. **94**(22): p. 12007-12012.

213. T.G. Burland, L. Solnica-Krezel, J. Bailey, D.B. Cunningham, and W.F. Dove, Adv. Microb. Physiol., 1993. **35**: p. 1-69.

214. M. Karl, R. Anderson, and E. Holler, Eur. J. Biochem., 2004. **271**(19): p. 3805-3811.

215. M. Karl, B. Gasselmaier, R.C. Krieg, and E. Holler, Eur. J. Biochem., 2003. **270**(7): p. 1536-1542.

216. A. Schmidt, C. Windisch, and E. Holler, Eur. J. Cell Biol., 1996. **70**(4): p. 373-380.

217. B. Angerer and E. Holler, Biochemistry (Mosc). 1995. **34**(45): p. 14741-14751.

218. S. Doerhoefer, S. Khodyreva, I.V. Safronov, W.A. Wlassoff, R. Anarbaev, O.I. Lavrik, and E. Holler, Microbiology, 1998. **144 (Pt 11)**: p. 3181-3193.

219. E. Holler, G. Achhammer, B. Angerer, B. Gantz, C. Hambach, H. Reisner, B. Seidel, C. Weber, C. Windisch, C. Braud, and et al., Eur. J. Biochem., 1992. **206**(1): p. 1-6.

220. G. Achhammer, A. Winkler, B. Angerer, and E. Holler, Curr. Genet., 1995. **28**(6): p. 534-545.

221. B. Willibald, W. Bildl, B.S. Lee, and E. Holler, Eur. J. Biochem., 1999. **265**(3): p. 1085-1090.

222. E. Holler, *Poly(malic acid) from Natural Sources*, in *Handbook of Engineering Polymeric Materials*, N.P. Cheremisinoff, Editor. 1997, Marcel Dekker: New York. p. 93-103.

223. J.A. Portilla-Arias, M. Garcia-Alvarez, A.M. de Ilarduya, E. Holler, J.A. Galbis, and S. Munoz-Guerra, Macromol. Biosci., 2008a. **8**(6): p. 540-550.

224. J.A. Portilla-Arias, M. Garcia-Alvarez, A.M. de Ilarduya, and S. Munoz-Guerra, Macromol. Biosci., 2007. **7**(7): p. 897-906.

225. T. Göttler and E. Holler, Biochem. Biophys. Res. Commun., 2006. **341**(4): p. 1119-1127.

226. M.E. Martinez Barbosa, S. Cammas, M. Appel, and G. Ponchel, Biomacromolecules, 2004. **5**(1): p. 137-143.

227. O. Thioune, H. Fessi, J.P. Devissaguet, and F. Puisieux, Int. J. Pharm., 1997. **146**(2): p. 233-238.

228. N. Belcheva, J. Smid, N. Lambov, N. Danchev, P. Peikov, A. Zlatkov, and C. Tsvetanov, J. Controlled Release, 1995. **37**(1-2): p. 43-48.

229. J.A. Portilla-Arias, M. Garcia-Alvarez, A. Martinez de Ilarduya, E. Holler, and S. Munoz-Guerra, Biomacromolecules, 2006. **7**(1): p. 161-170.

230. G. Heun, N. Lambov, A. Zlatkov, P. Peikov, I. Doytchinova, and K. Gesheva, J. Controlled Release, 1999. **58**(2): p. 189-194.

231. N. Lambov, P. Peikov, N. Belcheva, K. Gesheva, A. Zlatkov, and N. Danchev, J. Controlled Release, 1997. **45**(2): p. 193-197.

232. K. Byrappa, S. Ohara, and T. Adschiri, Adv. Drug Delivery Rev., 2008. **60**(3): p. 299-327.

233. M.J. Cocero, A. Martin, F. Mattea, and S. Vaona, J. Supercrit. Fluids, 2009. **47**: p. 546-555.

234. K. Mishima, Adv Drug Deliv Rev, 2008. **60**(3): p. 411-432.

235. E. Franceschi, A.M. De Cesaro, M. Feiten, S.R.S. Ferreira, C. Dariva, M.H. Kunita, A.F. Rubira, E.C. Muniz, M.L. Corazza, and J.V. Oliveira, J. Supercrit. Fluids, 2008. **47**: p. 259-269.

236. P. Chattopadhyay, R. Huff, and B.Y. Shekunov, J. Pharm. Sci., 2006. **95**(3): p. 667-679.

Chapter I C

Introduction

Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/nano-beads in biotechnological and biomedical applications

Katrin Grage[1], Anika C. Jahns[1], Natalie Parlane[2], Rajasekaran Palanisamy[1], Indira A. Rasiah[1], Jane A. Atwood[1] and Bernd H. A. Rehm[1,*]

[1]Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand, and [2]Hopkirk Research Institute, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand

* Corresponding author: Bernd H. A. Rehm, e-mail: b.rehm@massey.ac.nz,

phone: +64 6 350 5515 ext. 7890, fax: +64 6 350 5688

Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/nano-beads in biotechnological and biomedical applications

Katrin Grage, Anika C. Jahns, Natalie Parlane, Rajasekaran Palanisamy, Indira A. Rasiah, Jane A. Atwood and Bernd H. A. Rehm

Abstract

Polyhydroxyalkanoates (PHAs) are naturally occurring organic polyesters that are of interest for industrial and biomedical applications. These polymers are synthesized by most bacteria in times of unbalanced nutrient availability from a variety of substrates and they are deposited intracellularly as insoluble spherical inclusions or PHA granules. The granules consist of a polyester core, surrounded by a boundary layer with embedded or attached proteins that include the PHA synthase, phasins, depolymerizing enzymes, and regulatory proteins. Apart from ongoing industrial interest in the material PHA, more recently there has also been increasing interest in applications of the PHA granules as nano-/micro-beads after it was conceived that fusions to the granule associated proteins (GAPs) provide a way to immobilize target proteins at the granule surface. This review gives an overview of PHA granules in general, including biogenesis and GAPs, and focuses on their potential use as nano-/micro-beads in biotechnological and biomedical applications.

Introduction

Bacterial polyhydroxyalkanoate (PHA) granules, which are found as naturally occurring spherical inclusions, are becoming increasingly recognized as potential functionalized beads for use in biotechnological and biomedical applications.

PHAs are polyesters which serve as carbon and energy storage for bacteria and become deposited as insoluble spherical inclusions in the cytoplasm. Most bacterial genera and even members of the family *Halobacteriaceae* of the *Archaea* are known to synthesize PHA[1-6] which is produced in conditions of nutrient limitation but where carbon is

available in excess[7-10]. Bacteria are able to accumulate as much as 80% of their dry weight in PHA[11, 12], with reversal of the PHA polymerisation process in conditions of carbon starvation[13, 14]. One of the most common PHAs is poly(3-hydroxybutyrate) (PHB) which is synthesized from 3-hydroxybutyrate (3HB), but different bacteria use hydroxy fatty acids of varying chain length, generating a range of PHAs.

Due to properties such as biocompatibility, biodegradability and production from renewable resources, there is considerable interest in the potential applications of PHAs. With chemical modification or through the creation of copolymers, a range of material properties can be achieved, for example, PHAs that are less brittle and more flexible while retaining tensile strength. These polymers have been developed for use in industrial or medical applications and have been shown to be well tolerated by mammalian systems.[15] Due to the comparatively high production costs, PHAs are currently mainly attractive for use in the medical field, for example, for sutures or implants like heart valves, stents and bone scaffolding.[15, 16]

The key enzyme for PHA biosynthesis is the PHA synthase. This enzyme polymerizes (R)-3-hydroxyacyl-CoA thioester monomers into polyester with the release of coenzyme A. Depending on the organism, there are several classes of PHA synthases using different (R)-3-hydroxyacyl-CoA precursors which can be provided by different pathways.[17] In *Cupriavidus necator*, the most investigated PHB producer,[18] *(R)*-3-hydroxybutyryl-CoA monomers are generated from acetyl-CoA by the action of two other enzymes[16, 19, 20]. The three PHB biosynthesis genes are organized in one operon, the *phaCAB* operon. *β*-Ketothiolase (encoded by *phaA*) condenses two molecules of acetyl-CoA to acetoacetyl-CoA and this is subsequently reduced to *(R)*-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (encoded by *phaB*). The PHB synthase (encoded by *phaC* in *C. necator*) then converts the thioester monomers into the polyoxoester PHB. The polymer aggregates to form a spherical inclusion or granule of usually 50 - 500 nm in diameter with the amorphous hydrophobic PHA polyester at the core and attached or embedded proteins at the surface, including the PHA synthase, PHA depolymerases, structural, and regulatory proteins.[21, 22]

In this review, we summarize the current literature on PHA granules, their biogenesis and structure, and on protein engineering approaches of associated proteins aiming at the design of PHA granules as biobeads for use in various biomedical applications.

Structure of PHA Granules

The structure of PHA granules has not been fully determined but the major constituent of granules is PHA, often PHB, with small amounts of protein and lipid.[23] *In vivo* the hydrophobic polyester core is largely amorphous,[24] with water as a component that prevents crystallization by acting as a plasticizer[25]. This is the mobile state of PHA, that is, the form that is subject to the action of synthesizing and degrading enzymes. After isolation, PHA is often crystalline (see below).

Initial studies, including electron microscopy in the 1960s,[26] have shown the polyester core to be surrounded by a 4 nm boundary layer, which most likely comprises a phospholipid monolayer[27] with embedded and attached proteins[22, 28]. While most data seem to be consistent with a monolayer, alternative membrane models, for example, comprising inner and outer protein layers sandwiching phospholipids, have been suggested.[29] More recent electron microscopy data indicated that the thickness of the surface layer surrounding the PHA granules to be 14 nm, which the authors took as an indication of the size of the associated proteins.[30] However, it cannot entirely be ruled out that the boundary layer primarily consists of proteins and that attachment of membrane material is only an isolation artefact.

In addition to EM, a variety of techniques have been used to investigate PHA, including wide-angle X-ray scattering,[31] nuclear magnetic resonance spectroscopy,[32] and confocal microscopy[30] (Figure 1). Using wide-angle X-ray scattering, Kawaguchi and Doi confirmed that PHA in native granules is amorphous, even after isolation, and that certain treatments seemed to initiate crystallization, presumably by removing a lipid component.[31] Recently, contrast-variation small-angle neutron scattering was used to probe granule organization, and results were consistent with the phospholipid monolayer model.[33, 34] Atomic force microscopy (AFM) allows imaging at nanoscale while being rapid and less damaging to preparations than EM.[35, 36] Recently, analysis of PHA granules by AFM has shown an additional network layer with globular areas, most likely also incorporating structural phasin proteins.[37] AFM was also used to show porin-like structures in the surrounding membrane, which were suggested to provide a portal to the amorphous polymer core and be the site of PHA metabolism and depolymerisation.[38]

Investigation of PHA granules is strongly influenced by the preparation technique because denaturation and crystallization of PHA often occurs through physical stress such as excessive sonication,[37] freeze-thaw cycles, or exposure to solvents, detergents, or alkalis[39]. To avoid denaturation during the purification process, PHA granules can be purified using mechanic (e.g., French Press) or enzymatic cell lysis followed by density gradient centrifugation. These techniques should allow accurate analysis and consistent end-use of PHA granules.
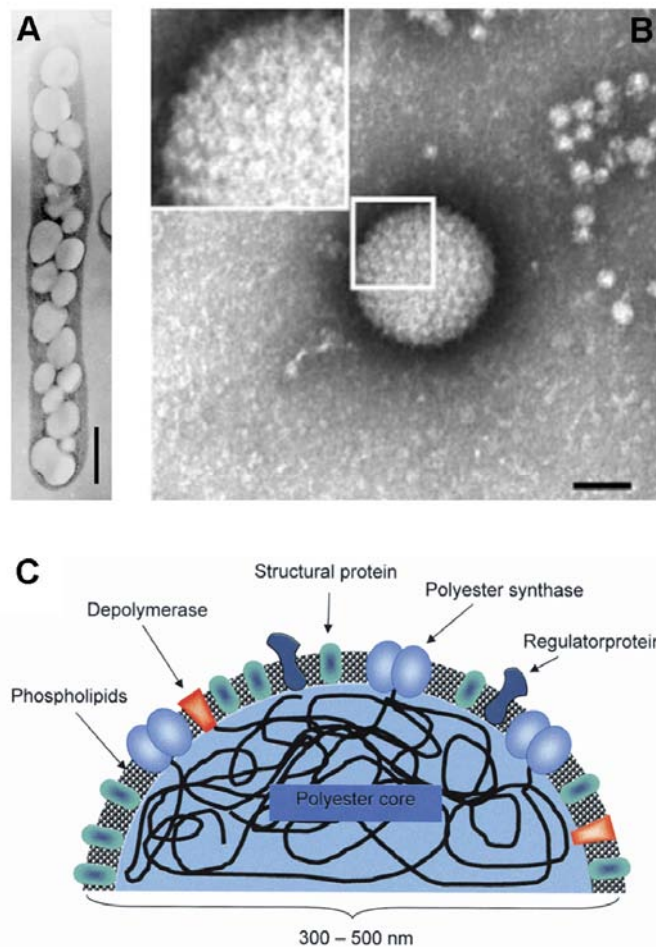
Figure 1. Different representations of PHA granules. (A) TEM image of *C. necator* cell filled with PHA granules. Bar, 500 nm. (*Journal of Bacteriology*, 2005, 187, 3814-3824, doi:10.1128/JB.187.11.3814-3824.2005 [54], reproduced with permission from American Society for Microbiology.) (B) TEM image of single PHA granule isolated from *C. latum* zooming in on the paracrystalline-like layer of particles covering the granule. Bar, 50 nm. (*Applied and Environmental Microbiology*, 2007, 73, 586-593, doi:10.1128./AEM.01839-06 [30], reproduced with permission from American Society for Microbiology.) (C) Schematic depiction of a PHA granule with granule associated proteins. (Reproduced with permission, from Rehm, B. H. A., 2003, *Biochemical Journal*, 376, 15-33, DOI 10.1042/BJ20031254 [58]. © the Biochemical Society.)

PHA Granule Assembly

The process of PHA biosynthesis, the polymerization of (*R*)-3-hydroxyacyl-CoA to PHA, leads to the formation of spherical inclusions, which start to assemble as the PHA synthase converts soluble substrate monomers into insoluble high molecular weight polymer. Two different models have been discussed to explain this process, which will be described below. During the polymerization process, the synthase remains covalently attached to the growing polyester chain and continues to incorporate more substrate until metabolic or spatial constraints terminate the polymerization procedure, that is, the substrate has been depleted or all available space in the cell has been used. The size of PHA granules and the number of inclusions per cell seem to vary between organisms (with the diameter usually ranging between 100 and 500 nm diameter and 5-10 granules per cell) and it has been debated if fusions between granules occur or if they are successfully prevented by the granule associated proteins (GAPs), in particular, the phasin PhaP[22, 40]. PhaP also impacts on the granule surface to volume ratio and thus the number of granules per cell[40, 41] and on PHA synthase activity[42, 43] (see below).

Apart from the metabolic background (which determines the provision of suitable substrate), factors which could potentially influence the molecular weight of the polymer include the PHA synthase concentration and presence of PHA depolymerising enzymes. The latter are only present in the native host and there are indeed indications that recombinant production yields higher molecular weight polymer than the native system.[44] An inverse correlation between PHA synthase concentration and molecular weight has so far only been shown for *in vitro* as well as recombinant production in *E. coli* and not for production in the native host.[45-47]

The exact mechanism by which the nascent polyester chains with attached synthesizing enzymes (enzyme-nascent polyester units) congregate to form the boundary layer-covered PHA granule has not been elucidated. Two models for granule biogenesis have mainly been discussed.[48] The first one is the "micelle" model, which is based on the assumption that the PHA synthase is present in the cell as a soluble enzyme, more or less randomly distributed in the cytoplasm. Once polymerization starts, the nascent polyester chain converts the initially soluble enzyme into an amphipathic molecule and the increasingly hydrophobic PHA chains aggregate into a micelle-like structure. In this model, the constituents of the boundary layer, that is, phospholipids and other GAPs

apart from the synthase, would gradually become incorporated as the self-assembled PHA inclusion increases in size. The second model is the more recent "budding" model, which suggests that the PHA synthase localizes to the inner face of the cytoplasmic membrane, either inherently or as soon as a PHA chain emerges from the enzyme. In this case, biosynthesis of the polyester would be directed into the intermembrane space where the extending chains would accumulate until eventually PHA inclusions surrounded by a phospholipid monolayer would bud off the membrane. While the "micelle" model is supported by the fact that PHA granules can be produced *in vitro* in the absence of membranes,[45, 49, 50] most of the recently emerging evidence is in favor of the "budding" model.

Jendrossek *et al*. analyzed early stages of PHB accumulation *in vivo* in *Rhodospirillum rubrum*, *C. necator,* and in recombinant *E. coli* by confocal laser scanning fluorescence microscopy (CLSM), visualizing PHB granules by Nile red staining and fusion of EYFP (enhanced yellow fluorescent protein) to a phasin.[51] In young cultures in the initial stages of PHB production, they observed PHB granules predominantly at or near the cell poles and near the cell wall. Jendrossek similarly analyzed *Caryophanon latum* by CLSM in combination with Nile red staining and by TEM in combination with immunogold staining and found that PHB granules in early stages of formation were localized close to the cytoplasmic membrane.[30] Also, in 2005, Peters and Rehm reported their fluorescence microscopy studies of emerging PHA granules in *Pseudomonas aeruginosa* PAO1 and recombinant *E. coli,* employing either the PHA synthase from *P. aeruginosa* or *C. necator,* which were both fused to GFP (green fluorescent protein) at their N terminus.[52] In both organisms, nascent PHA granules were observed to localize to the cell poles and occasionally to the poles and to the center of the cell, that is, to the future cell poles. This localization occurred independently of septum formation but seemed to require proper nucleoid structure and segregation as was demonstrated by analysing PHA granule formation in a *mukB* mutant, which is affected in nucleoid structure and segregation. This study suggested that nucleoid occlusion, that is, spatial competition between nucleoid and PHA granules might play a role in intracellular localization of granule formation. This was the first evidence that the cytoskeleton might be involved in PHA granule formation.[52] Further investigations revealed no evidence that the nascent polyester chain is responsible for subcellular localization of the synthase (e.g., by anchoring it to the membrane), as even an inactive mutant of the *C. necator* PHB synthase still localized to the cell poles.[53] The

deletion of either the N or the C terminus of the synthase, respectively, did also not affect proper positioning. These results indicate that the core region of the *C. necator* PHB synthase might be responsible for polar localization. The observations of Jendrossek *et al.* and Peters *et al.* provide support for the budding model as early stage PHA granules were localized at the cell poles and thus (a) close to the membrane and (b) not randomly distributed. However, these findings might also indicate that the situation is more complex as according to the model one would expect PHA granules to emerge along the circumference of the entire cell, not only at the poles.

Tian *et al.* have suggested a third model based on their kinetic studies of PHB granule biogenesis in *C. necator* by TEM.[54] They observed dark-stained elongated structures or "mediation elements" in the center of the cell with small granules attached. In older cultures, these elements were no longer visible, which could either mean that they had been degraded or that they were covered by granules. The authors proposed these mediation elements to serve as scaffolds for the initiation of granule formation, which would be analogous to the cellulosome. Although the observations of Tian *et al.* are currently not compatible with the polar localization of nascent PHA granules described by other researchers as described above, they also support a non-random distribution.

Another question is how synthesis of the PHA chain is terminated. In particular, does it happen in a way which enables the PHA synthase to reinitiate synthesis and generate more than one polymer chain? Calculations addressing this question have mainly been based on data derived from *in vitro* PHA biosynthesis. Based on the amount and molecular weight of the PHA produced *in vitro* by different organisms, it was calculated that both the PHA synthase from *C. necator* (class I) and from *P. aeruginosa* (class II) synthesized not more than one polyester chain per molecule of enzyme, while the *Allochromatium vinosum* synthase (class III) produced multiple chains.[42, 45, 49] Tian *et al.* made an attempt to determine this ratio for the *in vivo* situation in *C. necator* and obtained a ratio of PHB molecules to PHB synthase molecules of 60 to 1.[47] Thus, some indications for chain transfer have been obtained in single cases but no definite conclusions could be drawn so far. Comparison of the molecular weight of PHA produced from different carbon sources led to the suggestion that some of them might act as chain transfer agents in chain termination and that the actual chain transfer agent *in vivo* might be 3-hydroxybutyric acid, which is not enzyme bound.[55]

Granule-Associated Proteins

Proteins associated with the phospholipid granule surface play a major role in PHA synthesis and degradation and in granule formation.[56] These proteins have been designated to four classes (names in brackets for *C. necator*), namely the polyester or PHA synthases (PhaC), the depolymerases (PhaZ), regulatory proteins (PhaR) and phasins (PhaP) (Figure 1).

PHA synthase

The PHA synthase, which is the key enzyme of PHA biosynthesis, catalyzes the stereo-selective conversion of *(R)*-3-hydroxyacyl CoA thioester substrates to PHA, with the concomitant release of coenzyme A.[57, 58] The ongoing increase in the number of published bacterial genomes has resulted in a corresponding increase in the number of putative PHA synthases. Currently, the nucleotide sequences of at least 88 PHA synthases have been obtained, including two potential PHA synthase genes from the halobacterial species *Haloarcula marismortui* and *Haloferax mediterranei*.[2, 6]. Based on their primary structures, as well as the number of subunits and substrate specificity, PHA synthases have been assigned to four major classes.[58]

Class I and class II PHA synthases consist of only one type of subunit (PhaC) with molecular weights between 61 and 73 kDa.[59] The PHA synthases belonging to class I (e.g., *C. necator*) utilize *(R)*-3-hydroxy fatty acid substrates consisting of 3-5 carbon atoms and produce PHA composed of short length monomers (PHA$_{SCL}$),[58, 60] whereas those of class II (e.g., *P. aeruginosa*) utilize *(R)*-3-hydroxy fatty acids with 6-14 carbon atoms and synthesize medium chain length PHA (PHA$_{MCL}$).[61, 62] PHA synthases of class III (e.g., *A. vinosum*) consist of two subunits, namely PhaC of 40 kDa with similarity to classes I and II polyester synthases and PhaE with no similarity to these, also of 40 kDa.[63, 64] Class IV PHA synthases, found in the genus *Bacillus*, also consist of two subunits, one being the 40kDa PhaC subunit and the other a 20kDa PhaR subunit.[65] The PHA molecules synthesised by the enzymes in classes III and IV are made of PHA$_{SCL}$. A small number of bacterial PHA synthases do not fit into the above classification.[66, 67] The archaeal PHA synthases investigated so far seem to be similar to class III enzymes.[2, 6]

Among the proteins associated with the granule surface, only the PHA synthase is required for PHA granule formation, in the presence of a suitable substrate. This not only allows *in vitro* synthesis,[45] it also makes recombinant production of PHA, for example, in *E. coli* relatively straightforward.[68] In addition, the PHA synthase stays covalently attached to the granule surface and tolerates N-terminal fusions with other proteins. Therefore, it is possible to engineer PhaC fusions for the immobilization and functional display of these proteins on the granule surface (see below).[69-72]

Comparison of the primary sequences of the PHA synthases has shown six conserved blocks and eight identical amino acids.[58] While the N-terminal region has no conserved sequences, this region may have a role in the level of PHA synthase expressed and in the yield of PHA.[73] The C-terminal region of approximately 40 amino acids is more conserved in classes I and II PHA synthases, consisting mainly of hydrophobic amino acids which suggests a role for this region in binding of the synthase to the hydrophobic granule core.[58] With regard to secondary structure, predictions from multiple sequence alignments have indicated that the PHA synthases mainly contain variable-loop and α-helical secondary structures.[74] The α/β hydrolase region, which has been shown to be essential for enzymatic activity[75] has been strongly suggested to exist in the C-terminal portion of the protein, based on a conserved domain homology search.[76] In addition, the presence of a conserved lipase-like box in the primary structure, where the catalytic site serine of the lipase is replaced by a cysteine in the PHA synthase (G-X-[S/C]-X-G), further indicates homology to lipases.[77] Three conserved amino acid residues (cysteine, aspartic acid, and histidine) are thought to be critical for the catalytic mechanism by forming a catalytic triad. PHA synthases exist in an equilibrium of monomeric and dimeric forms *in vitro*, however, when the *(R)*-3-hydroxyacyl-CoA substrate is provided, significant dimerization is suggested to occur, with one subunit of the active dimer attaching to the growing polyester chain while the other subunit binds a new *(R)*-3-hydroxyacyl CoA substrate molecule.[78] Evidence has also been presented by mutational studies of the PHA synthase of *A. vinosum*, that the conserved aspartic acid residue plays an important role in chain elongation, while digestion of the polyester chain-enzyme complex and HPLC analysis have shown that the polyester chain stays covalently attached to the conserved cysteine of the enzyme.[79, 80]

PHA depolymerases

PHA depolymerases, enzymes which degrade PHA, consist of two groups, namely the intracellular depolymerases that degrade the amorphous PHA within granules of the accumulating bacteria, and the extracellular depolymerases, which are secreted by most bacteria to utilize denatured PHA present in the environment from, for example, other non-living cells.[81] PhaZ refers to the intracellular depolymerases found on the PHA granule surface. These are necessary for the mobilization of the PHA granules as a source of energy.[14]

Intracellular depolymerases have been investigated much less than the extracellular depolymerases and the mechanism by which intracellular native PHA granules can be reutilized is not well understood. There are some studies which have addressed the mobilization of intracellular PHA and the PhaZ encoding genes of *C. necator*.[14, 82-84] While the first described PhaZ of *C. necator* was designated PhaZ1,[83] subsequent (putative) depolymerases have been identified in *C. necator* and designated PhaZ2 to PhaZ5.[84-86] The genome sequence of *C. necator* revealed seven genes for PHA depolymerase isoenzymes and two for PHA oligomer hydrolases,[87] but for few there is actual direct evidence for their *in vivo* function. Following an alternative nomenclature, these putative depolymerases have also been designated PhaZa1 to PhaZa5, PhaZb, PhaZc and PhaZd1/2.[88]

Saegusa *et al.* reported the cloning and sequencing of the intracellular *phaZ* of *C. necator* and the demonstration of PHA degrading activity when amorphous PHA granules were provided as the substrate.[83] Although PHB metabolism has been reported to be cyclic in nature with PHB synthesis and degradation occurring at the same time,[82, 89] it has been a matter of discussion that simultaneous synthesis of PHB from acetyl-CoA and degradation of PHB to 3HB would be a waste of energy.[83] Very recently, Uchino *et al.* presented evidence that the PHA depolymerase PhaZa1 from *C. necator* is responsible for the degradation of PHB granules, albeit not exclusively, and that the enzyme degrades the polymer by thiolysis into 3HB-CoA instead of 3HB which would help to explain the previously apparently futile cycle of simultaneous PHB biosynthesis and degradation.[88, 90]

PHA depolymerases investigated in bacteria other than *C. necator* include the recently described PhaZ of *Pseudomonas putida* and *Azotobacter chroococcum*.[91, 92]

Phasins

Phasins are the most abundant protein found at the PHA granule surface and are synthesized in very large quantities under storage conditions, representing as much as 5 % of the total cellular protein.[40, 93-96] Phasins are noncatalytic proteins, consisting of a hydrophobic domain, which associates with the PHA granule surface and a predominantly hydrophilic domain exposed to the cytoplasm of the cell. There is evidence that this amphiphilic layer of phasins stabilizes PHA granules and prevents coalescence of separated granules.[40, 94, 95]

Phasins are low-molecular-weight proteins (mostly between 11 and 25 kDa) and have been identified and isolated from many PHA_{SCL}-producing bacteria due to their association with PHA granules.[40, 93-97] Phylogenetically, phasins are non related and share no sequence homology.[93] The phasin protein of *Rhodococcus ruber* binds to the PHA granule surface via two hydrophobic domains at the C terminus of the protein.[95, 97] In contrast, no distinct region in the PhaP1 protein from *C. necator* could be identified to be responsible for the binding of this protein to the granule surface.[93, 98] Therefore, binding capacity due to secondary or maybe also tertiary and quaternary structure of the protein has been suggested.[98] PhaP1 of *C. necator* is the most investigated member of this class of proteins. Genome analysis of *C. necator* identified three PhaP homologues which need to be further investigated.[85] Initial studies confirmed that PhaP1 is the major phasin protein and was recently characterized as a planar triangular protein that occurs as trimer.[98] Mutants defective in one of the *phaP1* homologues did not show any differences in phenotype compared to the wild type.[21] Phasins are not essential for PHA accumulation, but strains unable to produce any phasin protein accumulate only one single large PHA granule, taking up all available space in the cell.[40] Overproduction of PhaP leads to the formation of many small granules.[41] The influence on granule size has been demonstrated both *in vivo* and *in vitro*.[49, 95, 96, 99, 100] Although phasins are not necessarily required for PHA production their synthesis and abundance is closely correlated to PHA accumulation.[10] Phasins are only produced under accumulating conditions[40, 41, 101] and the amount of protein produced parallels the level of PHA in the cell.[43, 47, 101, 102] Additionally, phasins are thought to positively influence synthase activity although no evidence for direct interaction was reported so far.[42, 49, 103, 104] The occurrence of phasins on the granule surface obviously prevents other proteins not related to PHA metabolism from binding in an unspecific manner to the PHA granule surface which could be disadvantageous for the overall metabolism of the cell.[40] In the

absence of PhaP this protective function can be partially resumed by other phasin like proteins such as BSA[42, 49] or HspA[105].

Recombinantly produced PhaP1 protein from *C. necator* was shown to be able to bind to triacylglycerol (TAG) inclusions in *Rhodococcus opacus* and *Mycobacterium smegmatis*, indicating the capability of PhaP to bind to any type of hydrophobic inclusion, irrespective of the compound stored in the core of the inclusion.[106]

First crystals of the phasin protein PhaP from *Aeromonas hydrophila* were obtained in 2006.[107]

Regulatory proteins

PHA granule synthesis and phasin production are tightly regulated by the effectiveness of the transcriptional regulator PhaR. Genes encoding proteins homologous to PhaR are widely distributed among PHA$_{SCL}$-producing bacteria, indicating an important role in the regulation of PHA$_{SCL}$ biosynthesis.[94, 95] So far, the PhaR proteins from *C. necator* and *Paracoccus denitrificans* have been further investigated and binding of the regulatory protein to DNA sequences upstream of the respective *phaP* and *phaR* genes could be shown for both organisms.[41, 108, 109] Additional evidence was derived from mutagenesis studies performed in *C. necator*, where no PhaP protein could be detected in a *phaC* deletion strain, whereas a *phaC/phaR* deletion strain as well as a *phaR* deletion strain synthesized large amounts of PhaP protein.[102] Deletion of *phaR* completely disconnected PhaP accumulation from PHB production in *C. necator*.[102] Based on these findings the following regulatory model has been suggested for *C. necator*:[41, 56, 102] Under conditions non-permissive for PHA biosynthesis, PhaR binds to the *phaP* promoter region and inhibits transcription. Under PHA-accumulating conditions, the PHA synthase starts synthesizing polyester chains, and PHA granules are formed. PhaR, with high binding capacity to hydrophobic surfaces, binds to the PHA granule surface, hence lowering the cytoplasmic concentration to a point too low to sufficiently repress the transcription of *phaP*. This leads to synthesis of PhaP, which immediately binds to PHA granules; no soluble PhaP is detectable in the cytoplasm. In later stages of the accumulation, when PHA granules reached the maximum size, most of the granule surface will be covered with PhaP protein, leaving no space for efficient PhaR binding. Increasing cytoplasmic concentration of PhaR again allows binding to the respective DNA sequences and repressing transcription of *phaP* and its own gene, indicating an efficient autoregulation of *phaR* expression to prevent synthesis of more

PhaR than is required for sufficient repression of *phaP* expression.[41, 56, 102] The same type of PhaP/PhaR regulation was found for *P. denitrificans*.[109] Additionally, simultaneous binding of PhaR to DNA and PHA granule surface *in vitro* as well as *in vivo* could be shown for this organism.[109, 110] These results indicated a bifunctional character for PhaR and implied that the protein has two separate domains for binding to the two molecules. Binding of PhaR to the PHA granule surface seems to be irreversible and mainly driven by nonspecific hydrophobic interactions, implying high affinity but low specificity. PhaR of *P. denitrificans* was the first regulatory protein reported to interact directly with PHA granules. In contrast to PHA binding, DNA binding is reversible and highly specific, presumably involving the N-terminal region of the PhaR protein, which shows high sequence homology among PhaR homologues.[110] Recent studies suggested PhaR to be a more global PHA-responsive repressor, involved not only in the expression of *phaP* but also in the expression of genes involved in other metabolic pathways.[102, 109, 111]

PhaF and PhaI have been reported to be granule-associated proteins with regulatory function for *Pseudomonas oleovorans* and a model similar to the *C. necator* PhaP/PhaR system has been suggested.[112] However, PhaR does not show sequence homology to PhaF or PhaI and, unlike PhaR, PhaF is also involved in the regulation of PHA synthase production in *P. oleovorans*.[112, 113]

Applications of PHA Granules

As mentioned briefly in the introduction, PHAs have been considered as bio-based and biodegradable alternatives to conventional petroleum-based plastics for over 20 years and have more recently, over approximately the last 10 years, attracted increasing interest for medical applications.[114-117] PHAs are used because of their biocompatibility, their modifiable physical and thermal properties and also because of their biodegradability, but they are generally used as a chemically extracted bulk material. Only very recently, researchers have started to exploit the particular spherical structure or "bead" nature of PHA granules. The general properties of PHA as a material in combination with the size and shell-core composition of PHA granules open up a broad range of applications in biotechnology and medicine, from protein purification to drug delivery (Figure 2).

Protein purification

Protein purification methods typically aim to recover a high yield of protein, free of contaminants, and without denaturing the biological activity. Consequently, separation methods must be sufficiently mild so as not to irreversibly alter the protein's structure. Techniques that meet these requirements include affinity-based methods that take advantage of bonding interactions between a protein analyte and an immobilizing matrix. These methods must be individually optimized for each protein which can be expensive and time consuming.[118] Producing the protein of interest fused to an affinity tag generally simplifies the purification procedure. Following purification of the protein the tag can be easily removed enzymatically. Although this approach is widely used and considered reliable for purifying the native target protein, the cost and number of separation steps involved can make the method cumbersome.[119] Recently, self-cleaving affinity tags based on inteins have been introduced to eliminate the need for expensive proteolytic enzymes.[120, 121] Despite the success of the self-cleaving affinity tagged purification process, the cost of these methods, especially the cost of affinity resins, and the relatively low binding capacity for the tagged protein prohibit large scale industrial protein purification.[122]

The large-molecular-weight spherical structure of PHA granules with the surface-associated proteins (PHA synthase, phasins, etc.) and the low-cost production make the granules a useful tool for protein immobilization and purification.[119, 123, 124] Banki *et al*. developed a protein purification system which combines two technologies, namely PHA production in recombinant *E. coli* and intein-mediated self-splicing, implementing the specific affinity of the *C. necator* phasin PhaP to the PHA granules.[122] In this system, the protein of interest is produced fused to the C terminus of PhaP which acts as an affinity tag. Both the tagged protein and the PHA granules are co-produced in *E. coli* and the protein binds to the granules, which act as an affinity matrix, via the phasin tag. After cell disruption, granules with bound protein can be separated from other cellular components by simple centrifugation. Following appropriate washing, the protein of interest is released by intein self-cleavage. Banki *et al*. used multiple phasins (2-3 repeats) and reported the successful purification of several test proteins (maltose binding protein (MBP), *β*-galactosidase (LacZ), chloramphenicol acetyltransferase (CAT) and NusA) with yields of 30-40 mg of protein per litre of culture.[122] The authors suggested that "fine-tuning", for example, of the granule size might further improve results.

Barnard *et al*. developed an analogous system for *C. necator* with the aim of overcoming the general disadvantages of using *E. coli* as a protein production host (e.g., inclusion body formation).[125] In addition, using a natural PHA producer has the advantage of having to recombinantly produce only one protein, the PhaP-tagged target protein. Barnard *et al*. demonstrated purification of GFP and LacZ from *C. necator* and further reported that PhaP is functional both as an N- and a C-terminal tag.

Recently, Wang *et al*. chose a slightly different approach to the same goal of PHA-based protein purification.[126] The protein production step was separated from the PHA production step and the protein purification procedure. While the target proteins (EGFP (enhanced GFP), MBP and LacZ) were tagged with *Aeromonas hydrophila* PhaP and recombinantly produced in *E. coli*, the PHA beads were produced *in vitro* from chemically extracted PHA bulk material. Following incubation of the PHA beads with protein crude extract, the target proteins were also retrieved by intein-mediated cleavage. This suggested that phasins do not only bind to emerging native granules but also to crystalline PHA. Moreover, the authors suggested one main advantage of their system, namely, suitability for a wider range of target proteins, including eukaryotic proteins, as the tagged protein can be produced in any host organism independent of PHA granule formation. One might also expect stronger target protein production if this process does not have to compete with PHA biosynthesis in the cell. On the other hand, compared to the methods of Banki *et al.* and Barnard *et al.* the method of Wang *et al.* requires more steps and also additional processing of the PHA. For all the PHA-based protein purification methods described here, one has to keep in mind that they are not suitable for the purification of proteins which themselves have a high affinity for PHA granules. In this case one would expect problems with separation and poor yield as the target proteins would compete with the phasins in binding to the granules.[127]

Banki *et al.* suggested that the self-contained system consisting of phasin tag and PHA affinity matrix is particularly suitable for large-scale purification with moderate purity requirements. Moldes *et al.* took an even simpler approach.[128] They used the N-terminal region of the *P. putida* phasin PhaF as a tag for protein purification and simply released the purified tagged protein (fusion of PhaF and target protein) by detergent treatment.

Biological Nano- /Micro-beads

The use of nanoparticles in drug delivery, target specific therapy, molecular imaging, and as biomarkers or biosensors, in diagnosis and many other biomedical fields is increasing rapidly.[129, 130] Recently, it has been conceived that PHA granules have great potential for development towards these applications.

In 2005 it was demonstrated that the fusion of GFP to the N terminus of the PHA synthase did not affect PHA granule formation[52] which encouraged further studies to engineer the PHA synthase to enable immobilization of the enzyme β-galactosidase.[131] Immobilized β-galactosidase was stable for several months under various storage conditions. This proof-of-principle work showed that protein engineering of the PHA synthase to produce functionalized PHA granules could be a useful tool for developing biological nano-/micro-beads for various applications. The PHA synthase has the advantages of providing a covalent interaction with the granule as well as a simpler recombinant production system in which no other genes apart from the PHA biosynthesis genes have to be heterologously expressed.

In order to develop a system for purification of immunoglobulin G (IgG), Brockelbank *et al*. engineered the PHA synthase by fusing the IgG binding ZZ domain of protein A from *Staphylococcus aureus* to the PhaC N terminus.[69] The IgG binding capacity of the ZZ domain-displaying granules (ZZ-PHA granules) was confirmed by enzyme–linked immunosorbent assay (ELISA). ZZ-PHA granules enabled efficient purification of IgG from human serum and performed equally well compared to commercial protein A-Sepharose beads with regard to both purity and yield.[69] In another recent work an anti-β-galactosidase scFv (single-chain variable fragment of an antibody) was immobilized at the surface of PHA granules following the same principle of using PhaC as a self-assembly promoting fusion partner.[70] The scFv-displaying beads were successfully used for specific binding and elution of their antigen β-galactosidase. The functional display of the scFv was further assessed by a quantitative enzyme linked assay measuring β-galactosidase activity. Both approaches indicated the functional display of the antibody fragment at the bead surface which makes these scFv-displaying beads a potential tool for diagnostic or therapeutic applications.[70] The main advantage of this system is the simple one-step production as opposed to laborious multiple steps required for immobilization of antibodies using conventional methods.
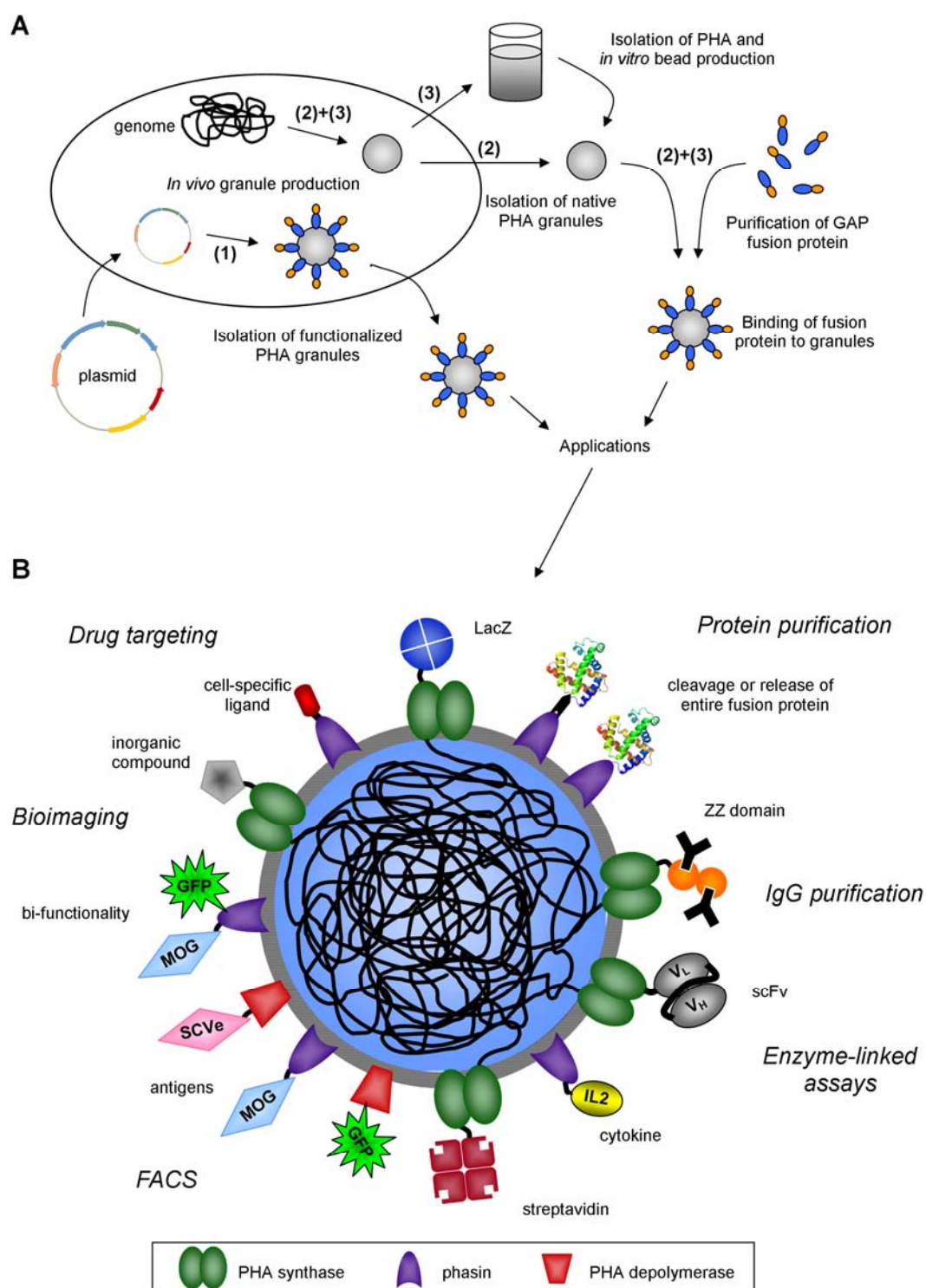
Figure 2. Potential applications for PHA granules. (A) Different approaches for the generation of functionalized PHA beads. (1) A plasmid encoded fusion of the target protein and a GAP is recombinantly produced in a PHA synthesizing host strain. (PHA synthesis can be natural or recombinant.) The fusion protein associates with PHA granules as they form and functionalized PHA granules are isolated from the cell. (2) and (3) Native PHA granules are formed by a natural PHA producing organism. This is either followed by isolation of these native granules (2) or by chemical extraction of the PHA and subsequent *in vitro* bead production (3). In a last step, the separately produced (and purified) GAP fusion protein is allowed to bind to the PHA granules/beads *in vitro* (2 and 3). (B) Schematic overview of the different proteins and other compounds which have so far been immobilized and functionally displayed at the PHA granule surface, pointing out potential applications.

The strong streptavidin-biotin bond can be used to attach various biomolecules to one another or onto a solid support. This is a powerful tool for purification or detection of these molecules. Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads was recently published by Peters and Rehm.[72] Different variants of streptavidin (mature full length, core and monomeric) were tested as C-terminal fusions to the PHA synthase, and the performance of the enzyme and the resulting streptavidin beads was analyzed. The PHA synthase retained its activity in all fusions, but the mature full length streptavidin performed best with regard to biotin binding. It was demonstrated that the *in vivo* generated streptavidin beads are applicable for ELISA, DNA purification, enzyme immobilization and flow cytometry.[72] In another study, Jahns *et al*. employed PHA granules as biological template structures for molecular biomimetics.[71] The PHA synthase was fused to genetically engineered proteins for inorganics (GEPIs) and additionally to the ZZ domain of *S. aureus*. This approach resulted in the production of PHA granules with a multifunctional surface displaying both specific binding sites for certain inorganic substances (gold or silica) and for IgG. These biobeads could serve as suitable tools for medical bioimaging procedures where an antibody-mediated targeted delivery of an inorganic contrast agent is desired.[71]

The examples of functionalized biobeads described so far are based on fusions to the PHA synthase and recombinant production mainly in *E. coli*. In order to expand the range of possible applications, the feasibility of displaying immunologically relevant eukaryotic proteins on the surface of the PHA granules was explored by Bäckström *et al*.[132] In this study, mouse myelin oligodendrocyte glycoprotein (MOG) and interleukin-2 (IL2) were individually immobilized at the granule surface *in vivo* in *E. coli* by generating fusions to the C terminus of PhaP.[132] Isolated beads displaying either MOG or IL2 were analyzed by fluorescence activated cell sorting (FACS) using monoclonal antibodies that recognize correctly folded MOG or IL2, respectively. Although both proteins are secreted proteins which normally form inclusion bodies when produced in the *E. coli* cytoplasm, they could be successfully produced in a properly folded state at the surface of PHA granules in this host. When an enterokinase recognition site was incorporated between PhaP and IL2, the latter could be cleaved off, demonstrating that the system enables purification of eukaryotic proteins. Moreover, the excellent long-term storage performance further supports the potential of these beads for diagnostic applications.[132]

In a follow-up proof-of-concept study, bifunctional PHA granules were generated which simultaneously displayed two protein-based functions suitable for FACS analysis.[133] GFP was either displayed fused to the N terminus of PhaC and MOG to the C terminus of PhaP or both proteins were fused to the N and C terminus of the phasin, respectively. This showed that bifunctional PHA nanobeads displaying e.g. a fluorescent protein and a protein with a specific interaction partner (antigen, receptor) could be used in diagnostics.

While most of the published reports on functionalised PHA nano- /micro-beads have involved protein engineering of the phasins or the PHA synthase, Lee *et al.* targeted the substrate binding domain (SBD) of the PHA depolymerase from *Alcaligenes faecalis*.[124] They reported the *in vitro* production of microbeads from extracted PHA to which separately synthesized GAP-tagged target proteins could subsequently be bound. Beads of 2μm diameter were generated and the model proteins EGFP, RFP (red fluorescent protein) and SARS-CoV (severe acute respiratory syndrome corona virus) envelope protein were immobilized at the surface via a fusion to the N terminus of the SBD. All model proteins, including the SARS-CoV envelope protein were successfully detected by FACS, which demonstrates the suitability also of this method for generating functionalized PHA beads for e.g. immunoassays or, as the authors suggested, the study of protein-protein interactions.

Targeted Drug Delivery

There is now general agreement based on a large amount of data that PHA-based medical devices are indeed well tolerated by the human body.[134, 135] Thus, functionalized PHA granules seem to be excellent candidates for targeted drug delivery as they combine the properties of a biocompatible polymer with the properties of a biobead. Though the *in vivo* PHA production system has the advantage of being relatively cost-effective compared to other drug carrier systems on the market, medical applications naturally require materials of extreme purity. Therefore, suitable methods would be needed for endotoxin removal from bacterially produced PHA bionanobeads.[16, 136] To date, the biocompatibility of PHA granules has not been specifically studied, but when considering the major constituent of the granules, the PHA, it is expected to be similar to the biocompatibility of PHA alone, pending the biological activity (toxicity, immunogenicity) of the surface proteins. Various drug

delivery and drug targeting systems based on biodegradable polymers are currently under development.[137, 138] Several of the functionalized PHA biobeads described above would be suitable for targeted drug delivery, but so far there has only been one report of *in vivo* animal tests[139].

Recently, Yao *et al.* exploited one of the *C. necator* phasins as a tag to develop a receptor-mediated drug delivery system.[139] PhaP was fused to the cell-specific ligands mannosylated human α1-acid glycoprotein (hAGP) and human epidermal growth factor (hEGF). hAGP is recognized by receptors on macrophages, hEGF by receptors on hepatocellular carcinoma cells. The fusion proteins were produced in *Pichia pastoris* and *E. coli*, respectively, purified and immobilized on *in vitro* generated and rhodamine B isothiocyanate (RBITC)-loaded PHA beads. Fluorescence microscopic examination showed that both ligand-PhaP-nanobeads were taken up by the correct type of cell *in vitro* and directed to the correct tissue in *in vivo* mouse experiments, demonstrating targeted delivery of the model drug RBITC.

Outlook

To date, a range of proteins and other molecules have been successfully immobilized at the surface of PHA granules, indicating that these bacterial storage compounds have potential to be developed into powerful tools for diagnostic and therapeutic biomedical applications. A particular advantage of PHA granules as functionalized nano-/micro-beads, apart from the simple and cost-effective production, is the oriented immobilization of, for example, proteins via the GAP-tag and thus high binding capacity of the resulting beads. Future work should include improved strategies for size control of *in vivo* produced beads as well as improved methods for pyrogen removal.

References

(1)   Brandl, H.; Gross, R. A.; Lenz, R. W.; Fuller, R. C. *Appl. Environ. Microbiol.*, **1998,** *54*, 1977-1982.

(2)   Han, J.; Lu, Q.; Zhou, L.; Zhou, J.; Xiang, H. *Appl. Environ. Microbiol.*, **2007,** *73*, 6058-6065.

(3)   Hezayen, F. F.; Rehm, B. H. A.; Eberhardt, R.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.*, **2000,** *54*, 319-325.

(4)   Hezayen, F. F.; Steinbüchel, A.; Rehm, B. H. A. *Arch. Biochem. Biophys.*, **2002,** *403*, 284-291.

(5)   Hezayen, F. F.; Tindall, B. J.; Steinbüchel, A.; Rehm, B. H. A. *Int. J. Syst. Evol. Microbiol.*, **2002,** *52*, 2271–2280.

(6)   Lu, Q.; Han, J.; Zhou, L.; Zhou, J.; Xiang, H. *J. Bacteriol.*, **2008,** *190*, 4173-4180.

(7)   Campisano, A.; Overhage, J.; Rehm, B. H. A. *J. Biotechnol.*, **2008,** *133*, 442-452.

(8)   Hoffmann, N.; Rehm, B. H. A. *Biotechnol. Lett.*, **2005,** *27*, 279-282.

(9)   Kim, Y., R.; Paik, H. J.; Ober, C. K.; Coates, G. W.; Batt, C. A. *Biomacromolecules*, **2004,** *5*, 889-891.

(10)   Kuchta, K.; Chi, L.; Fuchs, H.; Pötter, M.; Steinbüchel, A. *Biomacromolecules*, **2007,** *2*, 657-662.

(11)   Lee, S. Y. *Trends Biotechnol.*, **1996,** *14*, 98-105.

(12)   Madison, L. L.; Huisman, G. W. *Microbiol. Mol. Biol. Rev.*, **1999,** *63*, 21-53.

(13)   Gao, D.; Maehara, A.; Yamane; T.Ueda, S. *FEMS Microbiol. Lett.*, **2001,** *196*, 159-164.

(14)   Handrick, R.; Reinhardt, S.; Jendrossek, D. *J. Bacteriol.*, **2000,** *20*, 5916-5918.

(15)   Hazer, B.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.*, **2007,** *74*, 1-12.

(16)   Furrer, P.; Panke, S.; Zinn, M. *J. Microbiol. Methods*, **2007,** *69*, 206-213.

(17)   Rehm, B. H. A. *Biotechnol. Lett.*, **2006,** *28*, 207-213.

(18)   Reinecke, F.; Steinbüchel, A. *J. Mol. Microbiol. Biotechnol.*, **2009,** *16*, 91-108.

(19)   Peoples, O. P.; Sinskey, A. J. *J. Biol. Chem.*, **1989,** *264*, 15298-15303.

(20)   Slater, S. C.; Voige, W. H.; Dennis, D. E. *J. Bacteriol.*, **1988,** *170*, 4431-4436.

(21)   Pötter, M.; Müller, H.; Steinbüchel, A. *Microbiology*, **2005,** *151*, 825-833.

(22)   Steinbüchel, A.; Aerts, K.; Babel, W.; Follner, C.; Liebergesell, M.; Madkour, M. H.; Mayer, F.; Pieper-Fürst, U.; Pries, A.; Valentin, H. E.; Wieczorek, R. *Can. J. Microbiol.*, **1995,** *41*, 94-105.

(23)   Griebel, R.; Smith, Z.; Merrick, J. M. *Biochemistry (Mosc).* **1968,** *7*, 3676-3681.

(24)   de Koning, G. J. M.; Lemstra, P. J. *Polymer*, **1992,** *33*, 3292-3294.

(25)   Horowitz, D. M.; Sanders, J. K. M. *J. Am. Chem. Soc.*, **1994,** *116*, 2695-2702.

(26)   Ellar, D.; Lundgren, D. G.; Okamura, K.; Marchessault, R. H. *J. Mol. Biol.*, **1968,** *35*, 489-502.

(27)   Mayer, F.; Madkour, M. H.; Pieper-Fürst, U.; Wieczorek, R.; Liebergesell, M.; Steinbüchel, A. *J. Gen. Appl. Microbiol.*, **1996,** *42*, 445-455.

(28)   Mayer, F.; Hoppert, M. *J. Basic Microbiol.*, **1997,** *37*, 45-52.

(29)   Stuart, E. S.; Tehrani, A.; Valentin, H. E.; Dennis, D.; Lenz, R. W.; Fuller, R. C. *J. Biotechnol.*, **1998,** *64*, 137-144.

(30)   Jendrossek, D.; Selchow, O.; Hoppert, M. *Appl. Environ. Microbiol.*, **2007,** *73*, 586-593.

(31)   Kawaguchi, Y.; Doi, Y. *FEMS Microbiol. Lett.*, **1990,** *70*, 151-156.

(32)   Barnard, G. N.; Sanders, J. K. *J. Biol. Chem.*, **1989,** *264*, 3286-3291.

(33)   Russell, R. A.; Holden, P. J.; Garvey, C. J.; Wilde, K. L.; Hammerton, K. M.; Foster, L. J. *Physica B*, **2006,** *385-386*, 859-861.

(34)   Russell, R. A.; Holden, P. J.; Wilde, K. L.; Hammerton, K. M.; Foster, L. J. R. *J. Biotechnol.*, **2007,** *132*, 303-305.

(35)   Matsumoto, K.; Matsusaki, H.; Taguchi, K.; Seki, M.; Doi, Y. *Biomacromolecules*, **2002,** *3*, 787-792.

(36)   Sudesh, K.; Gan, Z.; Matsumoto, K. i.; Doi, Y. *Ultramicroscopy*, **2002,** *91*, 157-164.

(37)   Dennis, D.; Sein, V.; Martinez, E.; Augustine, B. *J. Bacteriol.*, **2008,** *190*, 555-563.

(38)   Dennis, D.; Liebig, C.; Holley, T.; Thomas, K. S.; Khosla, A.; Wilson, D.; Augustine, B. *FEMS Microbiol. Lett.*, **2003,** *226*, 113-119.

(39)   Griebel, R. J.; Merrick, J. M. *J. Bacteriol.*, **1971,** *108*, 782-789.

(40)   Wieczorek, R.; Pries, A.; Steinbüchel, A.; Mayer, F. *J. Bacteriol.*, **1995,** *177*, 2425-2435.

(41)   Pötter, M.; Madkour, M. H.; Mayer, F.; Steinbüchel, A. *Microbiology*, **2002,** *148*, 2413-2426.

(42)   Qi, Q.; Steinbüchel, A.; Rehm, B. H. A. *Appl. Microbiol. Biotechnol.*, **2000,** *54*, 37-43.

(43)   York, G. M.; Stubbe, J.; Sinskey, A. J. *J. Bacteriol.*, **2001,** *183*, 2394-2397.

(44)   Kusaka, S.; Abe, H.; Lee, S. Y.; Doi, Y. *Appl. Microbiol. Biotechnol.*, **1997,** *47*, 140-143.

(45)   Gerngross, T. U.; Martin, D. P. *Proc. Natl. Acad. Sci. U. S. A.*, **1995,** *92*, 6279-6283.

(46)   Sim, S. J.; Snell, K. D.; Hogan, S. A.; Stubbe, J.; Rha, C.; Sinskey, A. J. *Nat. Biotechnol.*, **1997,** *15*, 63-67.

(47)   Tian, J.; He, A.; Lawrence, A. G.; Liu, P.; Watson, N.; Sinskey, A. J.; Stubbe, J. *J. Bacteriol.*, **2005,** *187*, 3825-3832.

(48)   Stubbe, J.; Tian, J. *Nat. Prod. Rep.*, **2003,** *20*, 445-457.

(49)   Jossek, R.; Reichelt, R.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.*, **1998,** *49*, 258-266.

(50)   Rehm, B. H. A.; Qi, Q. S.; Beermann, B. B.; Hinz, H. J.; Steinbüchel, A. *Biochem. J.*, **2001,** *358*, 263-268.

(51)   Jendrossek, D. *Biomacromolecules*, **2005,** *6*, 598-603.

(52)   Peters, V.; Rehm, B. H. A. *FEMS Microbiol. Lett.*, **2005,** *248*, 93-100.

(53)   Peters, V.; Becher, D.; Rehm, B. H. A. *J. Biotechnol.*, **2007,** *132*, 238-245.

(54)   Tian, J.; Sinskey, A. J.; Stubbe, J. *J. Bacteriol.*, **2005,** *187*, 3814-3824.

(55)   Madden, L. A.; Anderson, A. J.; Shah, D. T.; Asrar, J. *Int. J. Biol. Macromol.*, **1999,** *25*, 43-53.

(56)   Pötter, M.; Steinbüchel, A. *Biomacromolecules*, **2005,** *6*, 552-560.

(57)   Grage, K.; Peters, V.; Palanisamy, R.; Rehm, B. H. A. In *Microbial production of biopolymers and biopolymer precursors*, Rehm, B. H. A., Ed. Caister Academic Press: 2009; pp 255-287.

(58)   Rehm, B. H. A. *Biochem. J.*, **2003,** *376*, 15-33.

(59)   Qi, Q.; Rehm, B. H. A. *Microbiology*, **2001,** *147*, 3353-3358.

(60)   Ren, Q.; De Roo, G.; Kessler, B.; Witholt, B. *Biochem. J.*, **2000,** *349*, 599-604.

(61)   Amara, A. A.; Rehm, B. H. A. *Biochem. J.*, **2003,** *374*, 413-421.

(62)   Qi, Q.; Rehm, B. H. A.; Steinbüchel, A. *FEMS Microbiol. Lett.*, **1997,** *157*, 155-162.

(63)   Liebergesell, M.; Schmidt, B.; Steinbüchel, A. *FEMS Microbiol. Lett.*, **1992,** *78*, 227-232.

(64)   Liebergesell, M.; Steinbüchel, A. *Eur. J. Biochem.*, **1992,** *209*, 135-150.

(65)   McCool, G. J.; Cannon, M. C. *J. Bacteriol.*, **2001,** *183*, 4235-4243.

(66)   Fukui, T.; Doi, Y. *J. Bacteriol.*, **1997,** *179*, 4821-4830.

(67)   Matsusaki, H.; Manji, S.; Taguchi, K.; Kato, M.; Fukui, T.; Doi, Y. *J. Bacteriol.*, **1998,** *180*, 6459-6467.

(68)   Lee, S. Y.; Lee, K. M.; Chan, H. N.; Steinbüchel, A. *Biotechnol. Bioeng.*, **1994,** *44*, 1337-1347.

(69)   Brockelbank, J. A.; Peters, V.; Rehm, B. H. A. *Appl. Environ. Microbiol.*, **2006,** *72*, 7394-7397.

(70)   Grage, K.; Rehm, B. H. A. *Bioconj. Chem.*, **2008,** *19*, 254-262.

(71)   Jahns, A. C.; Haverkamp, R. G.; Rehm, B. H. A. *Bioconj. Chem.*, **2008,** *19*, 2072-2080.

(72)   Peters, V.; Rehm, B. H. A. *J. Biotechnol.*, **2008,** *134*, 266-274.

(73)   Normi, Y. M.; Hiraishi, T.; Taguchi, S.; Abe, H.; Sudesh, K.; Najimudin, N.; Doi, Y. *Macromol. Biosci.*, **2005,** *5*, 197-206.

(74)   Cuff, J. A.; Clamp, M. E.; Siddiqui, A. S.; Finlay, M.; Barton, G. J. *Bioinformatics*, **1998,** *14*, 892-893.

(75)   Pham, T. H.; Webb, J. S.; Rehm, B. H. A. *Microbiology*, **2004,** *150*, 3405-3413.

(76)   Rehm, B. H. A. *Curr. Issues Mol. Biol.*, **2007,** *9*, 41-62.

(77)   Jia, Y.; Kappock, T. J.; Frick, T.; Sinskey, A. J.; Stubbe, J. *Biochemistry (Mosc).* **2000,** *39*, 3927-3936.

(78)   Jia, Y.; Yuan, W.; Wodzinska, J.; Park, C.; Sinskey, A. J.; Stubbe, J. *Biochemistry (Mosc).* **2001,** *40*, 1011-1019.

(79)   Tian, J.; Sinskey, A. J.; Stubbe, J. *Biochemistry (Mosc).* **2005,** *44*, 8369-8377.

(80)   Tian, J.; Sinskey, A. J.; Stubbe, J. *Biochemistry (Mosc).* **2005,** *44*, 1495-1503.

(81)   Jendrossek, D.; Handrick, R. *Annu. Rev. Microbiol.*, **2002,** *56*, 403-432.

(82)   Doi, Y.; Segawa, A.; Kawaguchi, Y.; Kunioka, M. *FEMS Microbiol. Lett.*, **1990,** *55*, 165-169.

(83)   Saegusa, H.; Shiraki, M.; Kanai, C.; Saito, T. *J. Bacteriol.*, **2001,** *183*, 94-100.

(84)   York, G. M.; Lupberger, J.; Tian, J. M.; Lawrence, A. G.; Stubbe, J.; Sinskey, A. J. *J. Bacteriol.*, **2003,** *185*, 3788-3794.

(85)   Pötter, M.; Müller, H.; Reinecke, F.; Wieczorek, R.; Fricke, F.; Bowien, B.; Friedrich, B.; Steinbüchel, A. *Microbiology*, **2004,** *150*, 2301-2311.

(86)   Schwartz, E.; Henne, A.; Cramm, R.; Eitinger, T.; Friedrich, B.; Gottschalk, G. *J. Mol. Biol.*, **2003,** *332*, 369-383.

(87)   Pohlmann, A.; Fricke, W. F.; Reinecke, F.; Kusian, B.; Liesegang, H.; Cramm, R.; Eitinger, T.; Ewering, C.; Pötter, M.; Schwartz, E.; Strittmatter, A.; Voss, I.; Gottschalk, G.; Steinbüchel, A.; Friedrich, B.; Bowien, B. *Nat. Biotechnol.*, **2006,** *24*, 1257-1262.

(88)   Uchino, K.; Saito, T.; Jendrossek, D. *Appl. Environ. Microbiol.*, **2008,** *74*, 1058-1063.

(89)   Taidi, B.; Mansfield, D. A.; Anderson, A. J. *FEMS Microbiol. Lett.*, **1995,** *129*, 201-205.

(90)   Uchino, K.; Saito, T.; Gebauer, B.; Jendrossek, D. *J. Bacteriol.*, **2007,** *189*, 8250-8256.

(91)   de Eugenio, L. I.; Garcia, P.; Luengo, J. M.; Sanz, J. M.; Roman, J. S.; Garcia, J. L.; Prieto, M. A. *J. Biol. Chem.*, **2007,** *282*, 4951-4962.

(92)   Saha, S. P.; Patra, A.; Paul, A. K. *J. Biotechnol.*, **2007,** *132*, 325-330.

(93)   Hanley, S. Z.; Pappin, D. J.; Rahman, D.; White, A. J.; Elborough, K. M.; Slabas, A. R. *FEBS Lett.*, **1999,** *447*, 99-105.

(94)   Liebergesell, M.; Steinbüchel, A. *Biotechnol. Lett.*, **1996,** *18*, 719-724.

(95)   Pieper-Fürst, U.; Madkour, M. H.; Mayer, F.; Steinbüchel, A. *J. Bacteriol.*, **1995,** *177*, 2513-2523.

(96)   Schultheiss, D.; Handrick, R.; Jendrossek, D.; Hanzlik, M.; Schüler, D. *J. Bacteriol.*, **2005,** *187*, 2416-2425.

(97)   Pieper-Fürst, U.; Madkour, M. H.; Mayer, F.; Steinbüchel, A. *J. Bacteriol.*, **1994,** *176*, 4328-4337.

(98)   Neumann, L.; Spinozzi, F.; Sinibaldi, R.; Rustichelli, F.; Pötter, M.; Steinbüchel, A. *J. Bacteriol.*, **2008,** *190*, 2911-2919.

(99)   Jurasek, L.; Marchessault, R. H. *Appl. Microbiol. Biotechnol.*, **2004,** *64*, 611-617.

(100)   Seo, M. C.; Shin, H. D.; Lee, Y. H. *Biotechnol. Lett.*, **2004,** *26*, 617-622.

(101)   York, G. M.; Junker, B. H.; Stubbe, J. A.; Sinskey, A. J. *J. Bacteriol.*, **2001,** *183*, 4217-4226.

(102)   York, G. M.; Stubbe, J.; Sinskey, A. J. *J. Bacteriol.*, **2002,** *184*, 59-66.

(103)   de Almeida, A.; Nikel, P. I.; Giordano, A. M.; Pettinari, M. J. *Appl. Environ. Microbiol.*, **2007,** *73*, 7912-7916.

(104)   Jurasek, L.; Marchessault, R. H. *Biomacromolecules*, **2002,** *3*, 256-261.

(105)   Tessmer, N.; König, S.; Malkus, U.; Reichelt, R.; Pötter, M.; Steinbüchel, A. *Microbiology*, **2007,** *153*, 366-374.

(106)   Hänisch, J.; Wältermann, M.; Robenek, H.; Steinbüchel, A. *Microbiology*, **2006,** *152*, 3271-3280.

(107)   Zhao, M.; Li, Z.; Zheng, W.; Lou, Z.; Chen, G. Q. *Acta Crystallograph. Sect. F Struct. Biol. Cryst. Commun.*, **2006,** *62*, 814-819.

(108)   Maehara, A.; Doi, Y.; Nishiyama, T.; Takagi, Y.; Ueda, S.; Nakano, H.; Yamane, T. *FEMS Microbiol. Lett.*, **2001,** *200*, 9-15.

(109)   Maehara, A.; Taguchi, S.; Nishiyama, T.; Yamane, T.; Doi, Y. *J. Bacteriol.*, **2002,** *184*, 3992-4002.

(110)   Yamada, M.; Yamashita, K.; Wakuda, A.; Ichimura, K.; Maehara, A.; Maeda, M.; Taguchi, S. *J. Bacteriol.*, **2007,** *189*, 1118-1127.

(111)   Povolo, S.; Casella, S. *Arch. Microbiol.*, **2000,** *174*, 42-49.

(112)   Prieto, M. A.; Bühler, B.; Jung, K.; Witholt, B.; Kessler, B. *J. Bacteriol.*, **1999,** *181*, 858-868.

(113)   Hoffmann, N.; Rehm, B. H. A. *FEMS Microbiol. Lett.*, **2004,** *237*, 1-7.

(114)   Chen, G. Q.; Wu, Q. *Biomaterials*, **2005,** *26*, 6565-6578.

(115)   Misra, S. K.; Valappil, S. P.; Roy, I.; Boccaccini, A. R. *Biomacromolecules*, **2006,** *7*, 2249-2258.

(116)   Sodian, R.; Sperling, J. S.; Martin, D. P.; Egozy, A.; Stock, U.; Mayer, J. E., Jr.; Vacanti, J. P. *Tissue Eng.*, **2000,** *6*, 183-188.

(117)   Williams, S. F.; Martin, D. P.; Horowitz, D. M.; Peoples, O. P. *Int. J. Biol. Macromol.*, **1999,** *25*, 111-121.

(118)   Freitag, R.; Horváth, C. In *Downstream processing, biosurfactants, carotenoids*, Barzana, E., Ed. Springer-Verlag: 1996; pp 17-59.

(119)   Banki, M. R.; Wood, D. W. *Microb. Cell Fact.*, **2005,** *4*.

(120)   Chong, S. R.; Mersha, F. B.; Comb, D. G.; Scott, M. E.; Landry, D.; Vence, L. M.; Perler, F. B.; Benner, J.; Kucera, R. B.; Hirvonen, C. A.; Pelletier, J. J.; Paulus, H.; Xu, M. Q. *Gene*, **1997,** *192*, 271-281.

(121)   Gillies, A. R.; Mahmoud, R. B.; Wood, D. W. In *High throughput protein expression and purification*, Doyle, S. A., Ed. Springer-Verlag: 2009; pp 173-183.

(122)   Banki, M. R.; Gerngross, T. U.; Wood, D. W. *Protein Sci.*, **2005,** *14*, 1387-1395.

(123)   Anderson, A. J.; Dawes, E. A. *Microbiol. Rev.*, **1990,** *54*, 450-472.

(124)   Lee, S. J.; Park, J. P.; Park, T. J.; Lee, S. Y.; Lee, S.; Park, J. K. *Anal. Chem.*, **2005,** *77*, 5755-5759.

(125)   Barnard, G. C.; McCool, J. D.; Wood, D. W.; Gerngross, T. U. *Appl. Environ. Microbiol.*, **2005,** *71*, 5735-5742.

(126)   Wang, Z.; Wu, H.; Chen, J.; Zhang, J.; Yao, Y.; Chen, G.-Q. *Lab on a Chip*, **2008,** *8*, 1957-1962.

(127)   Maehara, A.; Ueda, S.; Nakano, H.; Yamane, T. *J. Bacteriol.*, **1999,** *181*, 2914-2921.

(128)   Moldes, C.; Garcia, P.; Garcia, J. L.; Prieto, M. A. *Appl. Environ. Microbiol.*, **2004,** *70*, 3205-3212.

(129)   Panyam, J.; Labhasetwar, V. *Adv. Drug Del. Rev.*, **2003,** *55*, 329-347.

(130)   Salata, O. *J. Nanobiotechnology*, **2004,** *2*, 3.

(131)   Peters, V.; Rehm, B. H. A. *Appl. Environ. Microbiol.*, **2006,** *72*, 1777-1783.

(132)   Bäckström, B. T.; Brockelbank, J. A.; Rehm, B. H. A. *BMC Biotechnol.*, **2007,** *7*, 3.

(133)   Atwood, J. A.; Rehm, B. H. A. *Biotechnol. Lett.*, **2009**. *31*. 131-137

(134)   Martin, D. P.; Williams, S. F. *Biochem. Eng. J.*, **2003,** *16*, 97-105.

(135)   Park, S. J.; Lee, S. Y. *J. Bacteriol.*, **2003,** *185*, 5391-5397.

(136)   Zinn, M.; Witholt, B.; Egli, T. *Adv. Drug Del. Rev.*, **2001,** *53*, 5-21.

(137)   Langer, R. *Nature*, **1998,** *392*, 5-10.

(138)   Langer, R.; Tirrell, D. A. *Nature*, **2004,** *428*, 487-492.

(139)   Yao, Y.-C.; Zhan, X.-Y.; Zhang, J.; Zou, X.-H.; Wang, Z.-H.; Xiong, Y.-C.; Chen, J.; Chen, G.-Q. *Biomaterials*, **2008,** *29*, 4823-4830.

Chapter I

Introduction

Aims and Scope of the thesis

Aims and Scope of the thesis

Polyhydroxyalkanoates (PHAs) are receiving considerable attention because of their potential as renewable and biodegradable plastics. Polyhydroxybutyrate (PHB), a representative compound of the family of PHAs, has many potential applications in medicine, veterinary practice, and agriculture due to its biodegradability and biocompatibility. Moreover, PHB is also an intracellular storage compound that provides a reserve of carbon and energy for many microorganisms. The key enzyme in the process of PHB granule formation is the PHA synthase. The objective of this PhD project is to introduce additional attachment functionality by modifying this polymerising enzyme to bind different metals and non-metals and hence to establish a technology platform to create high value products. Genetically engineered peptides for binding inorganics (GEPIs) will be fused to the N terminus of the PHA synthase, and the ability of the fusion protein to bind gold or silica will be evaluated. The bio-beads will be tailored for potential biomedical applications.

Previous work has shown that the PHA synthase PhaC from *Ralstonia eutropha* can be genetically engineered at its N terminus without affecting the polymerising activity of this enzyme. The research of this thesis aims to investigate whether the C terminus of PhaC can be modified in the same way. The C terminus is thought to be essential for activity and therefore no engineering or modifying was attempted so far. In a first experiment the maltose binding protein MalE will be fused to the C terminus of the PHA synthase. Both proteins will be connected via a specially designed linker region to maintain the hydrophobic environment in the vicinity of the C terminus of the PHA synthase. It is our view that this might be important to ensure activity of this enzyme. Establishing a technique to manipulate the N as well as the C terminus of the PHA synthase would broaden the biotechnological applicability of the PHA beads displaying the engineered protein at the bio-bead surface.

Chapter II

The class I polyhydroxyalkanoate synthase from *Ralstonia eutropha* tolerates translational fusions to its C terminus: A new mode of functional display

Anika C. Jahns and Bernd H. A. Rehm*

Institute for Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

*Author for correspondence: Bernd H. A. Rehm, phone: +64 6 350 5515 ext 7890; fax: +64 6 350 2267; e-mail: b.rehm@massey.ac.nz

The class I polyhydroxyalkanoate synthase from *Ralstonia eutropha* tolerates translational fusions to its C terminus: A new mode of functional display

Anika C. Jahns and Bernd H. A. Rehm

Abstract

Here, the class I PHA synthase (PhaC) from *Ralstonia eutropha* was investigated regarding the functionality of its conserved C-terminal region and its ability to tolerate translational fusions to its C terminus. MalE, the maltose binding protein, and GFP, the green fluorescent protein were considered as reporter proteins to be translationally fused to the C terminus. Interestingly, only when a linker was inserted between PhaC and MalE, PhaC remained active, whereas MalE was not functional. However, the extension of the PhaC N terminus by 458 amino acid residues was required to achieve functionality of MalE. These data suggested a positive interaction of the extended N terminus with the C terminus. To assess whether a linker and/or N-terminal extension is in general required for a functional C-terminal fusion, the GFP was fused to the C terminus of PhaC. Both fusion partners were active without the requirement of a linker and/or N-terminal extension. A further reporter protein, the IgG binding ZZ domain of protein A was translationally fused to the N terminus of the fusion protein PhaC-GFP and resulted in a tripartite fusion protein mediating production of polyester granules displaying two functional protein domains.

Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters, synthesized by many bacteria and some archaea in times of unbalanced nutrient availability (7, 14-16, 22). These polyesters are stored as water-insoluble inclusions inside the cells and serve as energy and carbon storage (11, 29, 30). PHA synthases catalyse the stereo-selective conversion of (*R*)-3-hydroxyacyl-CoA to PHAs while CoA is released and intracellular PHA granules are formed (32). The PHA synthase remains covalently attached to the PHA

granule surface and has been targeted by protein engineering, i.e. translational fusion to the dispensable and variable N-terminus, to enable display of various protein functions without affecting the synthase activity (8, 26). PHA granules displaying certain functionalities have been considered as bio-beads for biotechnological and medical applications (11).

PHA synthases can be divided into four classes. Class I and class II enzymes consist of only one subunit (PhaC) (28) and produce short-chain-length PHAs (class I) or medium-chain-length PHAs (class II) respectively (30, 33). Polyester synthases belonging to class III consist of two subunits, PhaC and PhaE and produce short-chain-length PHAs (20, 21). Class IV PHA synthases are similar to enzymes belonging to class III. The synthases of this class comprise the two subunits PhaC and PhaR (23, 24). It has previously been shown that the N terminus of PhaC is a highly variable region and not essential for PHA synthase activity (30, 35). In contrast, the C terminus is a rather conserved region among class I and class II PHA synthases and is essential for enzyme activity (31). Alignments of the amino acid sequences of different PHA synthases revealed that the C terminus of these enzymes is hydrophobic and therefore suggested to interact with the hydrophobic core of PHA granules (30). The PhaC subunits of class III and class IV PHA synthases do not show a high hydrophobicity for their C-terminal regions. Previous studies showed that the PhaC subunit of the class IV PHA synthase from *Bacillus megaterium* tolerates fusions to its C terminus without loss in activity as long as the hydrophobic second subunit PhaR is present as well (23).

The aim of this study was to assess the effect on enzyme activity of the conserved hydrophobic C terminus of PhaC with regard to the possibility of translationally fusing protein functions for the display at the PHA granule surface. This will be of interest for the display of proteins that require their free C terminus for activity.

Materials and Methods

Bacterial strains, plasmids and oligonucleotides

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *E. coli* XL1 Blue was grown at 37°C, BL21(DE3) at 25°C. Antibiotics were added in the respective concentration, ampicillin 75 µg/ml, tetracycline 12.5 µg/ml or chloramphenicol 50 µg/ml.

Construction of plasmids encoding C-terminal synthase fusions

General cloning procedures and isolation of DNA were performed as described elsewhere (34). Deoxynucleoside triphosphate, T4 DNA ligase and Pfx polymerase were purchased from Invitrogen ™ (CA, USA), primers were purchased from Sigma-Aldrich (St. Louis, Mo., USA). DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using an automatic sequencer (Applied Biosystems, 3730 DNA Analyzer).

The nucleic acid sequence of *phaC* was PCR amplified using the oligonucleotides PhaC XbaI and PhaC no stop and the plasmid pCWE as template. The sequence of MalE was amplified from the vector pMAL-c2G, using the oligonucleotides MalE XhoI and MalE BamHI. The GFP sequence was amplified from plasmid pCWE$_{Spe}$GFP, using the oligonucleotides GFP no start XhoI and GFP stop BamHI. To obtain the sequences of the complete fusion proteins, all respective fragments were ligated step by step into the respective restriction enzyme sites of vector pET-14b. The two linker sequences were encoded in the respective forward and reverse primers and used for direct primer-dimer ligation (linker: VLAVIDKRGGGGG; SG-linker: SGGGSGGGSGGGGS). Hence, plasmids pET-14b PhaC-linker-MalE, pET-14b PhaC-linker-SG-linker-MalE, pET-14b PhaC-linker-GFP and pET-14b PhaC-linker-SG-linker-GFP were obtained. To construct a direct linker-free fusion the 3' part of *phaC* was amplified by PCR using the oligonucleotides AscI PhaC and XhoI PhaC no stop. The respective fragment was exchanged to create plasmids pET-14b PhaC-MalE and pET-14b PhaC-GFP. In order to further expose the MalE protein a non-functional N-terminal extension to PhaC was obtained. The sequence of this M extension was amplified from plasmid pMAL-Mpl-EC (gift from Dr Peilin Xu), and plasmid pCWE$_{Spe}$Mpl-EC was obtained. Further subcloning led to the plamids pET-14b M-PhaC-MalE, pET-14b M-PhaC-linker-MalE and pET-14b M-PhaC. Plasmid pET-14b ZZ-PhaC-GFP codes for the double functionalised synthase. The ZZ-fragment was subcloned from plasmid pET-14b ZZ(-)phaC and inserted into the respectively prepared plasmid pET-14b PhaC-GFP. All plasmids and oligonucleotides are listed in Table 1. The respective plasmids were used to transform competent *E. coli* BL21 (DE3) cells harbouring plasmid pMCS 69. The plasmid pMCS69 encodes the *β*-ketothiolase (*phaA*) and the acetoacetyl-CoA-reductase (*phaB*) which are required for the formation of the precursor *R*-3-hydroxybutyryl-CoA, the substrate of the polyester synthase (1).

Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study

E. coli strain, plasmid or oligonucleotide	genotype	Source or reference
	Strains	
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17(r-$_k$, m+$_k$), supE44, relA1, -, lac[F', proAB, lacI^q, lacZΔM15, Tn10(Tc^r)]*	(8)
BL21(DE3)	F-, *ompT, hsdS*$_B$ (r$_B^-$ m$_B^-$), *gal, dcm* (DE3)	Novagen
	Plasmids	
pMCS69	pBBR1MCS derivative containing genes *pha*A and *pha*B of *R. eutropha* colinear to *lac* promoter	(13)
pET-14b	Ar^r, T7 promoter	Novagen
pETC	pET-14b derivative, coding for PhaCwt under T7 promoter control	(22)
pCWE	pBluescriptSK- derivative containing the *Nde*I/*Bam*HI inserted *phaC* gene from *R. eutropha*	(21)
pMAL-c2G	Plasmid for expression and purification of maltose-binding protein fusions	New England Biolab
pCWE$_{Spe}$GFP	pCWE$_{Spe}$ containing *Spe*I-inserted *gfp* gene	(20)
pET-14b ZZ(-)phaC	pET-14b containing *Xba*I/*Bam*HI fragment comprising gene *ZZ-phaC*	(7)
pET-14b PhaC-linker-MalE	pET-14b derivative containing *malE* fused to the 3' end of *phaC* via a linker sequence	This study
pET-14b PhaC-MalE	pET-14b derivative containing *malE* directly fused to the 3' end of *phaC*	This study
pET-14b M-PhaC-linker-MalE	pET-14b PhaC-linker-MalE derivative containing the Mpl sequence fused to the 5' end of *phaC*	This study
pET-14b M-PhaC-MalE	pET-14b PhaC-MalE derivative containing the Mpl sequence fused to the 5' end of *phaC*	This study
pMAL-Mpl-EC	pMAL-c2x derivative, containing Mpl sequence	(30)
pCWE$_{Spe}$Mpl-EC	pCWE derivative containing the Mpl sequence fused to the 5' end of *phaC*	This study
pET-14b M-PhaC	pETC derivative, containing the Mpl sequence fused to the 5' end of *phaC*	This study
pET-14b PhaC-linker -SG-linker-MalE	pET-14b PhaC-linker-MalE derivative containing the SG-linker sequence upstream of *malE*	This study
pET-14b PhaC-linker-GFP	pET-14b derivative containing *gfp* fusded to the 3' end of *phaC* via a linker sequence	This study
pET-14b PhaC-linker -SG-linker-GFP	pET-14b PhaC-linker-GFP derivative containing the SG-linker sequence upstream of *gfp*	This study
pET-14b PhaC-GFP	pET-14b derivative containing *gfp* directly fused to the 3'end of *phaC*	This study
pET-14b ZZ-PhaC-GFP	pET-14b PhaC-GFP derivative containing the ZZ sequence 5' of *phaC*	This study
	Oligonucleotides	
linker I	5'-P-TAT GGT GCT GGC GGT GGC GAT TGA TAA ACG CGG AGG CGG TGG AGG CC-3'	This study
linker II	5'-P'TCG AGG CCT CCA CCG CCT CCG CGT TTA TCA ATC GCC ACC GCC AGC ACC A-3'	This study
MalE XhoI	5'-CTC GAG ATG AAA ATC GAA GAA GGT AAA CTG GTA ATC-3'	This study
MalE BamHI	5'-GGA TCC TTA CTT GGT GAT ACG AGT CTG CGC GTC TTT CAG GGC TTC ATC GAC-3'	This study
PhaC XbaI	5'-TCT AGA AAT AAG GAG ATA CTA GTA TGG CGA CCG GCA AAG GCG CGG CAG CTT CCA CGC AG-3'	This study
PhaC no stop	5'-CAT ATG TGC CTT GGC TTT GAC GTA TCG CCC AGG-3'	This study
GFP no start XhoI	5'-ATG ACC TCG AGA GTA AAG GAG AAG AAC TTT TCA CTG GAG TTG TC-3'	This study
GFP stop BamHI	5'-GGA TCC TCA TTT GTA TAG TTC ATC CAT GCC ATG TGT AAT-3'	This study
SG linker I	5'-P-TCG AGA GCG GCG GTG GCG GTA GCG GTG GCG GTG GCA GCG GCG GTG GCG GTA GCC CCG GGC-3'	(11), modified

SG linker II	5'-TCG AGC CCG GGG CTA CCG CCA CCG CCG CTG CCA CCG CCA CCG CTA CCG CCA CCG CCG CTC-3'	(11), modified
AscI PhaC	5'-GGC GCG CCG TGC GCG CTG CT-3'	This study
XhoI PhaC no stop	5'-CTC GAG TGC CTT GGC TTT GAC GTA T-3'	This study

PHA granule isolation, polyester and protein analysis

Polyhydroxybutyrate (PHB) granule isolation was carried out as described previously (18), the polyester production was confirmed by gas chromatography/mass spectrometry (GC/MS) (7). PHB granule protein profiles were analyzed by SDS-PAGE as described elsewhere (19). The gels were stained with Coomassie brilliant blue G250.

MALDI-TOF mass spectrometry

The bead proteins were separated by SDS-PAGE and protein bands of interest were cut off the gel. Proteins were subjected to MALDI-TOF mass spectrometry. Identification of tryptic peptides was performed by collision induced dissociation tandem mass spectrometry and enabled identification of fusion proteins.

Microscopy

Solution of cells containing PHB granules and isolated granules were examined for GFP fluorescence as described previously (25).

ELISA

Enzyme-linked immunosorbent assays were normalized according to the protein concentration, which was determined by Bradford (6). The wells of microtiter plates were coated with 100 µl of a granule suspension and incubated overnight at 4°C. After blocking with 3% (w/v) bovine serum albumine, each well was washed with phosphate buffered saline containing 0.05% Tween 20. The wells were then incubated with mouse monoclonal to maltose binding protein (HRP) conjugate (abcam, Cambridge, UK) for 1 h. After extensive washing 100 µl of an *o*-phenylenediamine substrate solution was added to each well, and after 15 min the reaction was stopped by adding 100 µl of 1 N H_2SO_4. The substrate conversion was measured at 490 nm using a microtiter plate reader (Biotek ELX808, Biostrategy, Auckland, NZ). The IgG binding ability was determined as described previously (18).

Maltose binding assay

PHB granule suspensions were normalized to protein concentration. 2 mg/ml protein was incubated with 0.6 mg/ml maltose, for 4 h at 25˚C, in small shaking Erlenmeyer flasks. The assay solution was centrifuged and the maltose concentration of the supernatant was determined using a colorimetric assay from Sigma (Sigma A3403).

Results

The specifically designed linker is required to allow translational fusions to the C terminus of the PHA synthase

E. coli BL21(DE3) cells harbouring the plasmids pMCS69 and pET-14b PhaC-MalE were not able to produce detectable amounts of PHB. However, cells containing the plasmids pMCS69 and pET-14b PhaC-linker-MalE produced PHB, as was confirmed by GC/MS analysis. The linker sequence was designed to maintain the hydrophobic environment close to the carboxy terminus of the synthase. Hydrophobic amino acid residues are followed by charged amino acid residues (VLAVIDKRGGGGG) to facilitate proper surface display of the fusion partner. Isolated PHB granules displayed the fusion protein, as shown by SDS-PAGE analysis and ELISA. Despite the detection of MalE by ELISA, no maltose binding could be shown using the maltose binding assay. Hence, the linker region between PhaC and MalE was further extended and the respective plasmid pET-14b PhaC-linker-SG-linker-MalE encoding the fusion protein was constructed. The second linker sequence was inserted behind the original linker and consisted of a repeated serine-glycine motive (SGGGSGGGSGGGGS) which was already previously successfully employed as linker between PhaC and a respective fusion partner (12). This plasmid also mediated PHB granule formation in recombinant *E. coli* (pMCS69) and the PHB granules displayed the fusion protein (Figure S1 in Supplemental Material). However, the activity of the fusion partner MalE could not be confirmed.

An N-terminal extension of the PHA synthase restores its activity when directly fused to MalE

A 458 aa long non-functional extension, designated M, of the N terminus of the synthase protein restored PHB granule forming activity even when the C terminus of

the synthase was directly fused to MalE. Cells harbouring the plasmid pET-14b M-PhaC-MalE produced detectable amounts of PHB in recombinant *E. coli* (pMCS69). The respective fusion protein was attached to the PHB granules (Figure S1 in Supplemental Material, ELISA data not shown) and identified by MALDI-TOF/MS analysis (data not shown). Although PhaC was active, activity of the fusion partner MalE could not be demonstrated with the maltose binding assay. A slightly modified hybrid gene encoding a fusion protein with the N-terminal extension and the linker sequence between PhaC and MalE mediated functionality of both fusion partners Cells harbouring plasmids pMCS69 and pET-14b M-PhaC-linker-MalE produced PHB granules, as was verified by GC/MS analysis. The fusion protein was identified by MALDI-TOF/MS (Table ST1 in Supplemental Material) and the isolated PHB granules displayed the fusion protein, as demonstrated by anti-MalE ELISA (Figure 1, Figure S1 in Supplemental Material). The maltose binding activity of the MalE protein was demonstrated using the maltose binding assay. These beads reduced the maltose concentration by up to 20%; control beads included in the experiment did not show any binding.
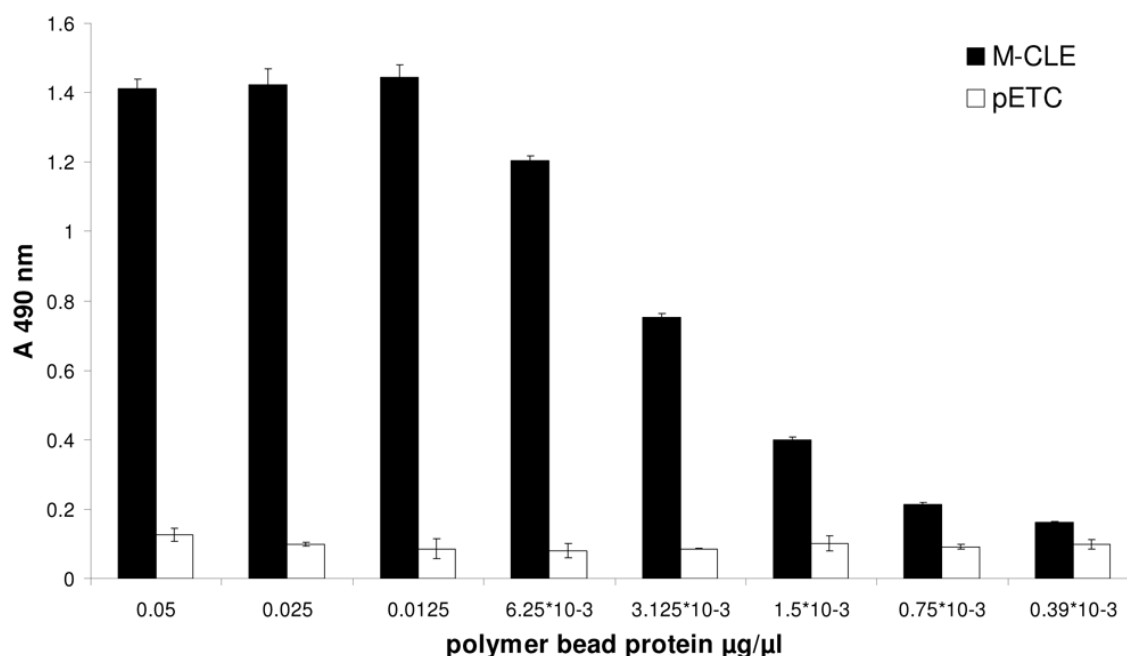
Figure 1. Display of the maltose binding protein assessed by ELISA. PHB granules were isolated from BL21(DE3)(pMCS69) harbouring pET-14b M-PhaC-linker-MalE (black columns) or pETC (white columns). All measurements were conducted in triplicates; standard deviations are indicated.

The activity of the entire fusion protein is dependent on the C terminal fusion partner

In order to assess whether the fusion protein data described above can be applied to various fusion partners, GFP was chosen as an alternative C-terminal fusion partner. Recombinant *E. coli* (pMCS69) harbouring the plasmid pET-14b PhaC-linker-GFP did not produce PHB, but cells showed non-localized green fluorescence, as observed by fluorescence microscopy (data not shown). Extending the linker region between both proteins resulted in detectable synthase and GFP activity. Recombinant *E. coli* (pMCS69) cells harbouring plasmid pET-14b PhaC-linker-SG-linker-GFP were able to synthesize PHB granules which displayed the fusion protein (Figure S2 in Supplemental Material). Green fluorescence of the isolated PHB beads as well as of cells containing granules displaying the fusion protein could be observed (data not shown). The fusion protein was identified by MALDI-TOF/MS (Table ST1 in Supplemental Material).

To assess whether a linker would be required for the functionality of both fusion partners, a direct fusion of the reporter protein GFP to the C terminus of the PHA synthase was constructed. This enabled the production of the fusion protein which was identified by MALDI-TOF/MS (Table ST1 in Supplemental Material). Recombinant *E. coli* (pMCS69) harbouring plasmid pET-14b PhaC-GFP produced PHB granules, as was confirmed by GC/MS analysis. SDS-PAGE analysis (Figure S2 in Supplemental Material) and fluorescence microscopy confirmed the display and functionality of the respective fusion protein at the granule surface (data not shown).

Translational fusions to the N and C terminus of the PHA synthase mediate simultaneous display of two protein function at the bead surface

After the successful engineering of the synthase C terminus for the display of one protein function, the possibility of the simultaneous display of two protein functions by generating one tripartite fusion protein was assessed. Therefore, plasmid pET-14b ZZ-PhaC-GFP was constructed and recombinant *E. coli* BL21 (pMCS69) harbouring this plasmid were able to produce PHB beads as verified by GC/MS. The fusion protein comprising the IgG-binding domain ZZ, the PHA synthase PhaC and the GFP protein was displayed at the polyester bead surface (Figure S3 and Table ST1 in Supplemental Material). These multifunctional PHB beads showed green fluorescence (data not shown) and were also able to bind IgG, as was shown by ELISA (Figure 2).
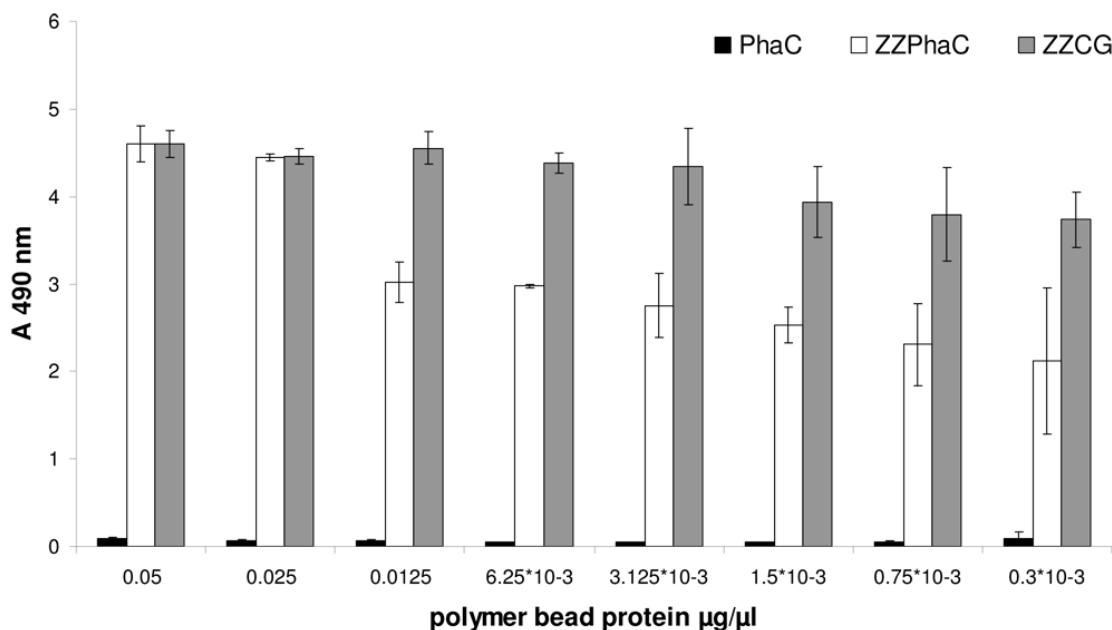
Figure 2. IgG binding ability of the tripartite synthase fusion protein assessed by ELISA. PHB granules were isolated from BL21(DE3)(pMCS69) harbouring either pETC (black columns), pET-14b ZZ(-)phaC (white columns) or pET-14b ZZ-PhaC-GFP (grey columns). All measurements were conducted in triplicates; standard deviations are indicated.

Discussion

This study provided insights into a new mode of functional display at the PHA granule surface, employing the C terminus of the class I PHA synthase as fusion target. The nature of the protein translationally fused to the C terminus of PhaC affects the activity of both fusion partners. Maintaining the hydrophobic environment around the C terminus of the synthase was found to be required to retain activity (30, 31). In contrast to N-terminal fusions some C-terminal fusions were inactivating the PHA synthase (Figure 3). MalE directly fused to the synthase C terminus led to inactivation, while a direct fusion of GFP to the C terminus of PhaC led to PHB granule formation and display of the functional fusion partner. A hydrophobicity analysis of the N-terminal regions of MalE and GFP showed that GFP comprises an extended hydrophobic region similar to the designed linker region (5, 10), whereas the N-terminal region of MalE was found to be hydrophilic. This suggested that the hydrophobic N terminus of GFP, when directly fused to PhaC, does not interfere with the proposed anchor function of the hydrophobic C terminus of the PHA synthase. However, MalE was shown to be compatible with various proteins when translationally fused to the N and C terminus of the respective protein (13). Intriguingly, the insertion of the designed linker between PhaC and GFP inactivated PhaC, whereas a further extension of the linker by an

additional SG-linker did not interfere with PHA synthase activity. These data implied that the N terminus of GFP provides inherently the hydrophobic environment needed by the synthase protein to remain active and the linker length inserted between PhaC and GFP is critical for PHA synthase activity.
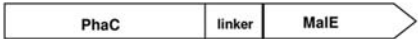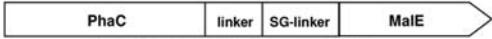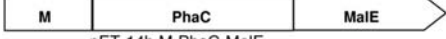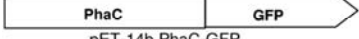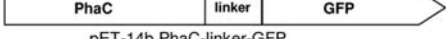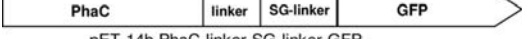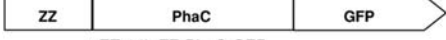

Hybrid Gene	PHB production	Activity of fusion partner	Surface display of fusion partner
PhaC — MalE pET-14b PhaC-MalE	-	-	-
PhaC — linker — MalE pET-14b PhaC-linker-MalE	+	-	+
PhaC — linker — SG-linker — MalE pET-14b PhaC-linker-SG-linker-MalE	+	-	+
M — PhaC pET-14b M-PhaC	+	-	N/A
M — PhaC — linker — MalE pET-14b M-PhaC-linker-MalE	+	+	+
M — PhaC — MalE pET-14b M-PhaC-MalE	+	-	+
PhaC — GFP pET-14b PhaC-GFP	+	+	+
PhaC — linker — GFP pET-14b PhaC-linker-GFP	-	+	+
PhaC — linker — SG-linker — GFP pET-14b PhaC-linker-SG-linker-GFP	+	+	+
ZZ — PhaC — GFP pET-14b ZZ-PhaC-GFP	+	+*	+*

Figure 3. Schematic overview of hybrid genes used in this study. PHB production was assessed by GC/MS analysis. The activity of the fusion partner was either assessed by fluorescence microscopy for GFP or maltose binding for MalE. The surface display of the fusion partner MalE was assessed by ELISA. * The IgG binding acitivity as well as the surface display of ZZ was assessed by ELISA.

Previously, the immobilization of proteins which needed a free carboxy terminus for activity, to the surface of PHA granules, was only possible when fused to the granule associated structural protein PhaP (2-4). In contrast to the phasin proteins, the PHA synthase, which is the only essential protein for granule formation, is covalently attached to the polyester granule surface. Therefore, utilizing the PhaC protein to fuse proteins of interest either N- or C-terminally, would allow a variety of proteins to be displayed on the PHA granule surface. In addition, simultaneous fusions to the N and C terminus, as shown with the tripartite fusion protein ZZ-PhaC-GFP, enables the display of two functionalities without the need for any additional protein anchor.

Acknowledgement

References

1. **Amara, A. A., and B. H. A. Rehm.** 2003. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochem. J. **374:**413-21.

2. **Atwood, J. A., and B. H. A. Rehm.** 2009. Protein engineering towards biotechnological production of bifunctional polyester beads. Biotechnol. Lett. **31:**131-7.

3. **Bäckström, B. T., J. A. Brockelbank, and B. H. A. Rehm.** 2007. Recombinant *Escherichia coli* produces tailor-made biopolyester granules for applications in fluorescence activated cell sorting: functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein. BMC Biotechnol. **7:**3.

4. **Banki, M. R., T. U. Gerngross, and D. W. Wood.** 2005. Novel and economical purification of recombinant proteins: intein-mediated protein purification using *in vivo* polyhydroxybutyrate (PHB) matrix association. Protein Sci. **14:**1387-95.

5. **Becker, K., M. Grey, and L. Bulow.** 2008. Probing protein surface accessibility of amino acid substitutions using hydrophobic interaction chromatography. J. Chromatogr. A **1215:**152-5.

6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-54.

7. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a Source of Poly(beta-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters. Appl. Environ. Microbiol. **54:**1977-1982.

8. **Brockelbank, J. A., V. Peters, and B. H. A. Rehm.** 2006. Recombinant *Escherichia coli* strain produces a ZZ domain displaying biopolyester granules suitable for immunoglobulin G purification. Appl. Environ. Microbiol. **72:**7394-7.

9. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-BLUE - A high-efficiency plasmid transforming RecA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376.

10. **Cserzo, M., E. Wallin, I. Simon, G. von Heijne, and A. Elofsson.** 1997. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng. **10:**673-6.

11. **Grage, K., A. C. Jahns, N. Parlane, R. Palanisamy, I. A. Rasiah, J. A. Atwood, and B. H. A. Rehm.** 2009. Bacterial Polyhydroxyalkanoate Granules: Biogenesis, Structure, and Potential Use as Nano-/Micro-Beads in Biotechnological and Biomedical Applications. Biomacromolecules **10:**660-669.

12. **Grage, K., and B. H. A. Rehm.** 2008. *In Vivo* Production of scFv-Displaying Biopolymer Beads Using a Self-Assembly-Promoting Fusion Partner. Bioconjug. Chem. **19:**254-62.

13. **Ha, J. S., J. J. Song, Y. M. Lee, S. J. Kim, J. H. Sohn, C. S. Shin, and S. G. Lee.** 2007. Design and application of highly responsive fluorescence resonance

energy transfer biosensors for detection of sugar in living *Saccharomyces cerevisiae* cells. Appl. Environ. Microbiol. **73:**7408-14.

14. **Han, J., Q. Lu, L. Zhou, J. Zhou, and H. Xiang.** 2007. Molecular characterization of the phaECHm genes, required for biosynthesis of poly(3-hydroxybutyrate) in the extremely halophilic archaeon *Haloarcula marismortui.* Appl. Environ. Microbiol. **73:**6058-65.

15. **Hezayen, F. F., B. H. A. Rehm, R. Eberhardt, and A. Steinbüchel.** 2000. Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. Appl. Microbiol. Biotechnol. **54:**319-25.

16. **Hezayen, F. F., A. Steinbüchel, and B. H. A. Rehm.** 2002. Biochemical and enzymological properties of the polyhydroxybutyrate synthase from the extremely halophilic archaeon strain 56. Arch. Biochem. Biophys. **403:**284-91.

17. **Hoffmann, N., A. A. Amara, B. B. Beermann, Q. Qi, H. J. Hinz, and B. H. A. Rehm.** 2002. Biochemical characterization of the *Pseudomonas putida* 3-hydroxyacyl ACP:CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. J. Biol. Chem. **277:**42926-36.

18. **Jahns, A. C., R. G. Haverkamp, and B. H. A. Rehm.** 2008. Multifunctional inorganic-binding beads self-assembled inside engineered bacteria. Bioconjug. Chem. **19:**2072-80.

19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-5.

20. **Liebergesell, M., B. Schmidt, and A. Steinbüchel.** 1992. Isolation and identification of granule-associated proteins relevant for poly(3-hydroxyalkanoic acid) biosynthesis in *Chromatium vinosum* D. FEMS Microbiol. Lett. **78:**227-32.

21. **Liebergesell, M., and A. Steinbüchel.** 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. Eur. J. Biochem. **209:**135-50.

22. **Lu, Q., J. Han, L. Zhou, J. Zhou, and H. Xiang.** 2008. Genetic and biochemical characterization of the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthase in *Haloferax mediterranei*. J. Bacteriol. **190:**4173-80.

23. **McCool, G. J., and M. C. Cannon.** 2001. PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. J. Bacteriol. **183:**4235-43.

24. **McCool, G. J., and M. C. Cannon.** 1999. Polyhydroxyalkanoate inclusion body-associated proteins and coding region in *Bacillus megaterium*. J. Bacteriol. **181:**585-92.

25. **Peters, V., D. Becher, and B. H. A. Rehm.** 2007. The inherent property of polyhydroxyalkanoate synthase to form spherical PHA granules at the cell poles: the core region is required for polar localization. J. Biotechnol. **132:**238-45.

26. **Peters, V., and B. H. A. Rehm.** 2005. In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS Microbiol. Lett. **248:**93-100.

27. **Peters, V., and B. H. A. Rehm.** 2008. Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads. J. Biotechnol. **134:**266-74.

28. **Qi, Q., and B. H. A. Rehm.** 2001. Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase. Microbiology **147:**3353-8.

29. **Rehm, B. H. A.** 2006. Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: The key role of polyester synthases. Biotechnol. Lett. **28:**207-13.

30. **Rehm, B. H. A.** 2003. Polyester synthases: natural catalysts for plastics. Biochem. J. **376:**15-33.

31. **Rehm, B. H. A., R. V. Antonio, P. Spiekermann, A. A. Amara, and A. Steinbüchel.** 2002. Molecular characterization of the poly(3-hydroxybutyrate) (PHB) synthase from *Ralstonia eutropha*: *in vitro* evolution, site-specific mutagenesis and development of a PHB synthase protein model. Biochim. Biophys. Acta **1594:**178-90.

32. **Rehm, B. H. A., and A. Steinbüchel.** 1999. Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. Int. J. Biol. Macromol. **25:**3-19.

33. **Ren, Q., G. De Roo, B. Kessler, and B. Witholt.** 2000. Recovery of active medium-chain-length-poly-3-hydroxyalkanoate polymerase from inactive inclusion bodies using ion-exchange resin. Biochem. J. **349:**599-604.

34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, NY.

35. **Schubert, P., N. Krüger, and A. Steinbüchel.** 1991. Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthetic operon: identification of the N terminus of poly(3-hydroxybutyrate) synthase and identification of the promoter. J. Bacteriol. **173:**168-75.

36. **Zhang, Q., R. M. Pan, Y. C. Ge, and P. Xu.** 2004. Expression of the soluble extracellular domain of human thrombopoietin receptor using a maltose-binding protein-affinity fusion system. Biol. Pharm. Bull. **27:**219-21.
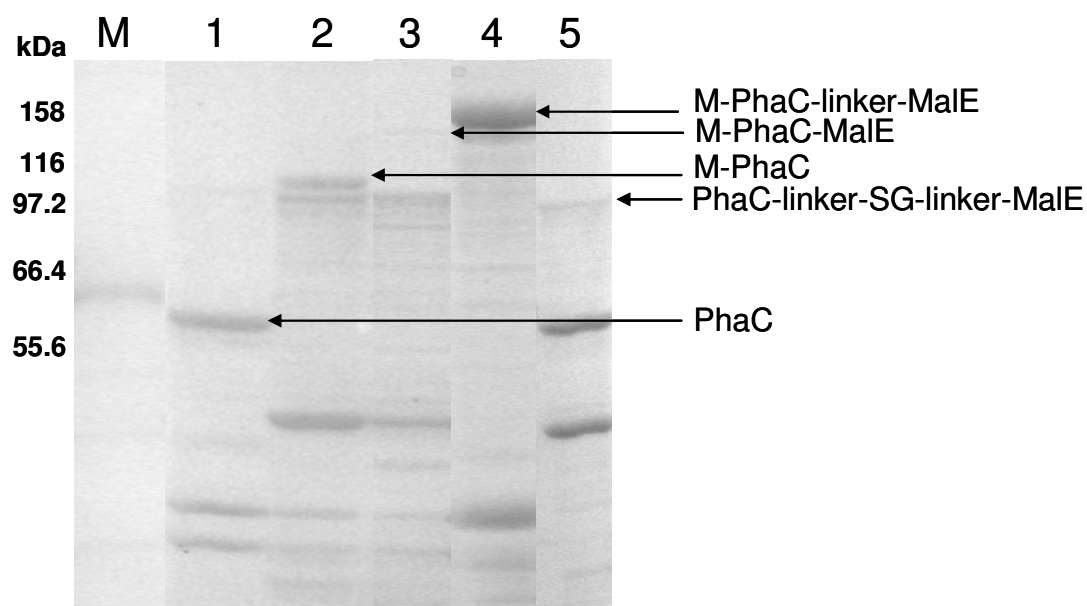
Supplemental Material

Figure S1. Protein profile of different PHB granules demonstrated by SDS-PAGE analysis. M, molecular mass standard (New England Biolabs, UK); lane 1, BL21(DE3)(pETC); lane 2, BL21(DE3)(pET-14b M-PhaC); lane 3, BL21(DE3)(pET-14b M-PhaC-MalE); lane 4, BL21(DE3)(pET-14b M-PhaC-linker-MalE); lane 5, BL21(DE3)(pET-14b PhaC-linker-SG-linker-MalE).
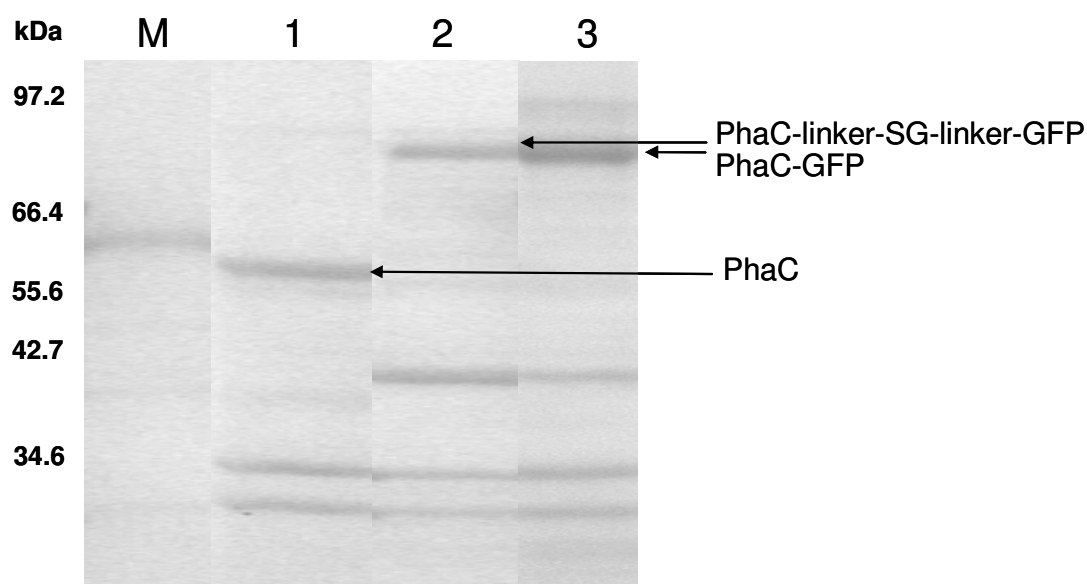

Figure S2. Protein profile of different PHB granules demonstrated by SDS-PAGE analysis. M, molecular mass standard (New England Biolabs, UK); lane 1, BL21(DE3)(pETC); lane 2, BL21(DE3)(pET-14b PhaC-linker-SG-linker-GFP); lane 3, BL21(DE3)(pET-14b PhaC-GFP).
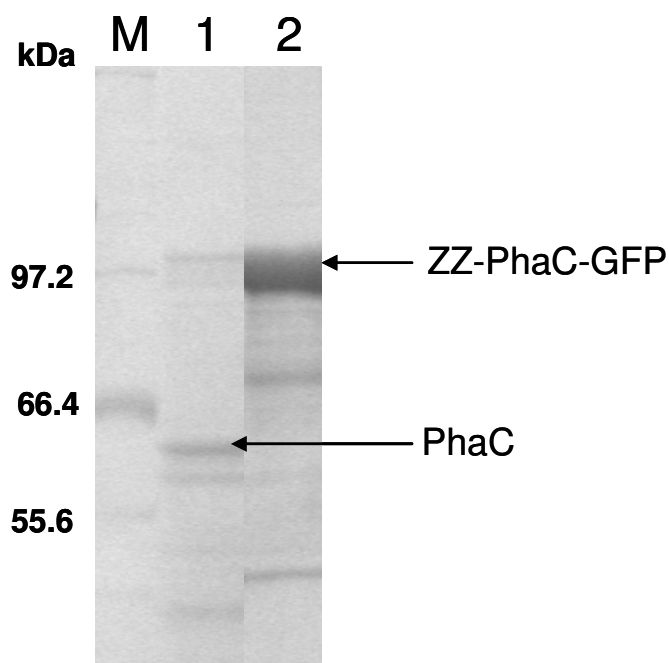
Figure S3. Protein profile of different PHB granules demonstrated by SDS-PAGE analysis. M, molecular mass standard (New England Biolabs, UK); lane 1, BL21(DE3)(pETC); lane 2, BL21(DE3)(pET-14b ZZ-PhaC-GFP).

Table ST1. Identified peptide fragments of protein analyzed by MALDI-TOF/MS

Protein	identified peptide fragments
PhaC-linker-SG-linker-GFP	V21-R36, I63-R74, T109-R125, F115-R125, A126-R141, F146-R169, L170-R180, G193-R207, I195-R207, N208-K234, Y250-R263, H264-R278, N279-R299, L456-R478, F501-K518, A564-R573, G630-K652, T723-R735
M-PhaC-linker-MalE	T21-R48, A53-R67, Y68-R79, A118-R143, E207-R234, L368-R376, L442-K464, V480-R495, I522-R533, F574-R584, F605-R628, L629-R639, I654-R666, N667-K693, Y709-R722, H723-R737, E830-R845, G846-K872, L915-R937, L1168-K1192, G1317-K1338, G1392-R1409
PhaC-GFP	I63-R74, T109-R125, F115-R125, A126-R141, L170-R180, L170-R184, G193-R207, R563-R573, A564-R573, Y574-K586, A576-K586, T687-R699
ZZ-PhaC-GFP	S149-R169, V154-R169, I196-R207, Y208-K223, A224-R234, F248-R258, A259-R274, I277-R302, F279-R302, L303-R313, G326-R340, I328-R340, N341-K367, Y383-R396, H397-R411, N412-R432, D439-R468, E504-R519, G520-K546, L589-R611, E612-K631, F634-K651, A697-R706, S809-R819, T820-R832, A833-R845, A833-K849, G850-K863, D939-K961

Chapter III

Multifunctional Inorganic-Binding Beads Self-Assembled Inside Engineered Bacteria

Anika C. Jahns[†], Richard G. Haverkamp[‡] and Bernd H. A. Rehm[†]*

[†]Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand; [‡]Institute of Technology and Engineering, Massey University, Private Bag 11222, Palmerston North, New Zealand

* Corresponding author: E-mail: b.rehm@massey.ac.nz. Phone: +64 6 350 5515 ext. 7890. Fax: +64 6 350 5688.

Multifunctional Inorganic-Binding Beads Self-Assembled Inside Engineered Bacteria

Anika C. Jahns, Richard G. Haverkamp and Bernd H. A. Rehm

Abstract

Multifunctional shell-core nano/micro-beads with a hydrophobic biopolymer core and a designed protein coat for selective binding of an inorganic substance and antibodies were self-assembled inside engineered bacteria. Hybrid genes were constructed to produce tailor-made bead-coating proteins in the bacterium *Escherichia coli*. These fusion proteins contained a binding peptide for an inorganic material, the antibody binding ZZ domain and a self-assembly promoting as well as biopolymer synthesizing enzyme. Production of these multi-domain fusion proteins inside *E. coli* resulted in self-assembly of beads comprising a biopolyester core and displaying covalently bound binding sites for specific and selective binding of an inorganic substance and any antibody belonging to the immunoglobulin G class. Engineered beads were isolated and purified from the respective *E. coli* cells by standard cell disruption procedures. Bead morphology and the binding functionalities displayed at the bead surface were assessed by the enzyme-linked immunosorbent assay, transmission electron microscopy, elemental analysis, backscattering electron density, analytical density ultracentrifugation, and atomic force microscopy. These analyses showed that bacteria can be engineered to produce fusion proteins mediating self-assembly of spherical biopolymer beads with binding affinity to gold and/or silica and antibodies. Spherical structures of this type could conceivably serve as nano/micro-devices for bioimaging in medical approaches where an antibody mediated targeted delivery of an inorganic contrast agent would be desired.

Introduction

Biological structures that specifically bind inorganics exert numerous potential biomedical applications. Therefore, the exploration of nature's structures for this purpose and the establishment of novel production methods is of great interest. In

particular, beads (nano- or micro-beads) made of biological and inorganic materials are suitable for a variety of applications such as, e.g., diagnostics (*1, 2*), imaging (*3*), and drug delivery (*4*). In binding assays, it was demonstrated that nanobeads displaying an antibody at high density show a higher binding affinity than the antibody itself enhancing the sensitivity of a diagnostic assay (*5*). Molecular biomimetic approaches have been used to specifically coat biological nanostructures, such as, e.g., self-assembling virus beads, with various inorganics for applications in imaging, biosensing, laboratory methods, and electronics (*6, 7*). Genetically engineered proteins for inorganics (GEPI) have been obtained using combinatorial approaches including phage display or cell surface display in order to select peptides binding inorganics (*8*). Peptides were isolated specifically binding (noble) metals, semiconducting oxides, and other technological compounds (*7-11*). This ultimately enabled the use of biological structures with protein components as templates for the defined nanoscale deposition of inorganics as well as control of geometrical shapes and sizes of inorganic nanobeads. This was achieved by engineering of proteins contributing to these structures enabling display of GEPIs for binding of inorganics (*7-11*). This resembles the morphogenesis in hard tissues (such as bones, dental tissues, mollusk shells, spicules) which is controlled by inorganic-binding proteins.

In this study, microbial polymer inclusions (50-300 nm) were conceived as biological template structures (*12-14*). These spherical inclusions are self-assembled inside the bacterial cell mediated by a single protein, the polyester synthase (PhaC) (*15, 16*). The polymer inclusions serve as reserve materials and comprise a hydrophobic polyester core surrounded by at least the PhaC protein (*17*). This natural polyester is composed of (*R*)-3-hydroxy fatty acids. Interestingly, PhaC catalyzes the core polymer synthesis as well as is required for the self-assembly. Moreover, PhaC has been found to remain covalently attached to the polymer core (*18, 19*). Further structural proteins (e.g., PhaP) have been found to specifically hydrophobically interact with the polymer core, but are not required for self-assembly (*15, 20-22*). Protein engineering of the synthase or structural proteins demonstrated that various functional proteins can be displayed at the polymer inclusions surface and that these inclusions can be stably maintained outside the cell as beads (*18, 23-28*). Here, it was demonstrated that genetic fusions of GEPIs to PhaC or previously engineered PhaC enabled production of beads that bind inorganic and biological molecules simultaneously. In this study, the antibody binding domain of protein A from *Staphylococcus aureus* was used as an additional binding functionality,

which ultimately enabled the production of spherical nano/micro-beads that could specifically bind not only any specific antibody but also an inorganic as suitable contrast agent applicable for, e.g., bioimaging approaches (*29*) (Figure 1). Moreover, the use of engineered *E. coli* as cell factory to produce functionalized beads in one step provides an efficient and economic production strategy.
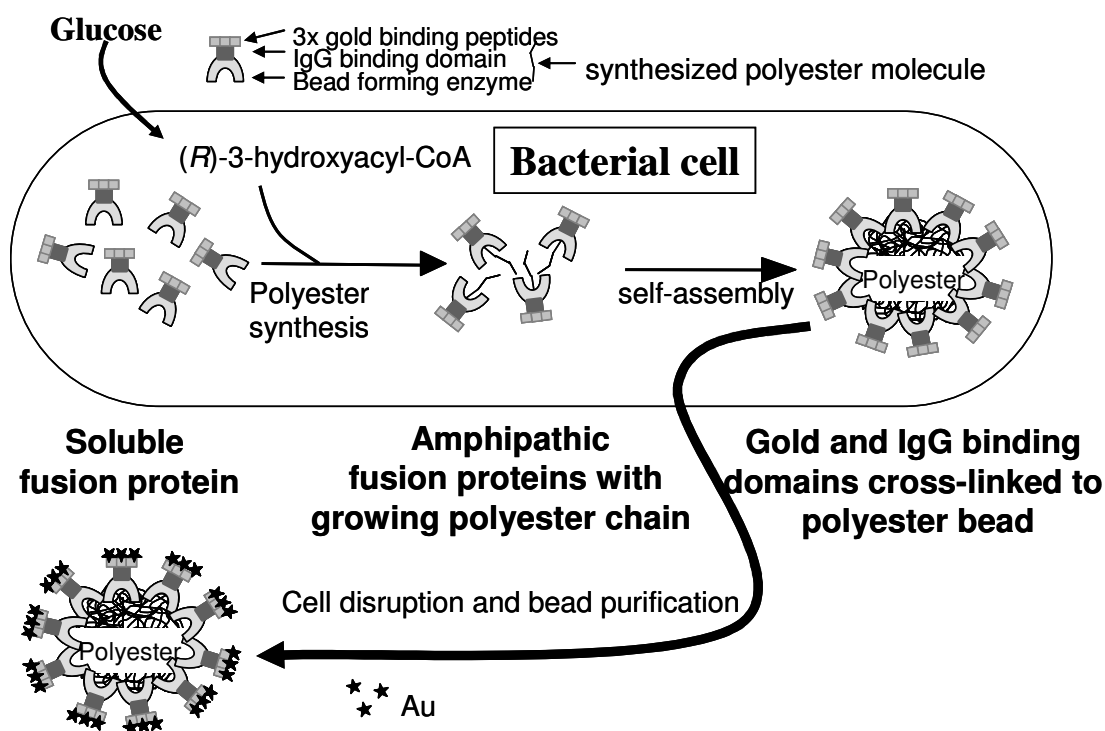

Figure 1. Schematic overview of polymer bead assembly.

Experimental Procedures

Bacterial Strains, Plasmids, Oligonucleotides, and DNA Constructs

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *E. coli* XL1-Blue was grown at 37°C, BL21(DE3) at 25°C. When required, ampicillin 75 µg/ml, tetracycline 12.5 µg/ml, or chloramphenicol 50 µg/ml were added. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

General cloning procedures and isolation of DNA were performed as described elsewhere (*30*). DNA primers, deoxynucleoside triphosphate, T4 DNA ligase and Taq polymerase were purchased from Invitrogen ™ (CA, USA). DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain

termination method using an automatic sequencer (Applied Biosystems, 3730 DNA Analyzer).

Gold-Binding Beads

To enable production of fusion proteins mediating production of polymer beads selectively binding gold, the GBP1 (*31*) encoding DNA sequence was inserted as a DNA fragment, composed of hybridized oligonucleotides GNI and GNII (Table 2), into the restriction enzyme sites *Xba*I/*Nde*I of plasmid pCWE. The resulting pCWE-GN-phaC contained the respective hybrid gene and was transformed into *E. coli* XL1-Blue harboring the plasmid pMCS69, which mediates synthesis of the polymer precursor required for bead production. A DNA sequence showing three repeats of the gold-binding peptide (*32*) was synthesized (DNA 2.0, Inc., CA, USA). The corresponding *Xba*I/*Sun*I fragment was inserted into plasmid pCWE, which was previously engineered to encode a pentaglycine linker and the ZZ domain of protein A both fused to the N terminus PhaC. The resulting plasmid pCWE-3xGN-G$_5$-ZZ-phaC was used to transform *E. coli* XL1Blue (pMCS69). To enable overproduction of the respective fusion protein, the corresponding hybrid gene was overexpressed under the control of the strong T7 promoter. Therefore, the hybrid genes were inserted into the *Xba*I/*Bam*HI site of vector pET-14b. The resulting plasmid pET-14b-3xGN-G$_5$-ZZ-phaC was used to transform *E. coli* BL21(DE3) harboring plasmid pMCS69.

Bead formation was assessed by analyzing the polyester constituting the bead core using gas chromatography-mass spectrometry as previously described (*16*). Moreover, beads formed inside the bacterial cell were stained with Nile red and observed using fluorescence microscopy as well as subjected to electron microscopy (*16*).

Silica-Binding Beads

To produce polymer beads with silica/antibody binding properties, the DNA sequence encoding the silica-binding peptide (*33*) encoding *Xba*I/*Nde*I DNA fragment, which was generated by hybridization of oligonucleotides SNI and SNII (Table 2), was ligated to the 5' end of *phaC* in plasmid pCWE. Together with pMCS69, this construct led to bead formation in *E. coli* XL1Blue. To achieve overproduction of the respective fusion proteins at the bead surface and thereby enhance the binding density, hybrid genes encoding fusion proteins were expressed under control of the strong T7 promoter. Therefore, the 1838 bp *Xba*I/*Bam*HI fragment was inserted into the respective sites of plasmid pET-14b and then transformed into *E. coli* BL21(DE3) cells harboring the plasmid pMCS69. A hybrid gene similar to the hybrid gene encoding the extended

gold-binding fusion protein was also constructed by using the oligonucleotides SN+I and SN+II (Table 2). This hybrid gene was inserted into plasmid pCWE which resulted in plasmid pCWE-SN-G$_5$-ZZ-phaC. The hybrid gene comprising the 2261 bp *Xba*I/*Bam*HI fragment was then subcloned into the respective sites of pET-14b to enable overexpression under T7 promoter control.

Multifunctional Beads

To synthesize polymer beads with gold, silica and antibody binding capability, the SN-G$_5$-ZZ(-)phaC sequence was amplified by PCR using oligonucleotides 3'PhaC-*Blp*I and *Bam*HI-SN+ (Table 2). The PCR product was confirmed by DNA sequencing and ligated into *Bam*HI/*Blp*I site of plasmid pET-14b-3xGN-G$_5$-ZZ(-)phaC which resulted in plasmid pET-14b-3xGN-G$_5$-ZZ(-)phaC/SN-G$_5$-ZZ(-)phaC. This new plasmid was used to transform competent *E. coli* BL21(DE3) cells harboring pMCS69 and to mediate production of multifunctional beads.

Table 1. Bacterial Strains, Plasmids, and Oligonucleotides Used for the Generation of Hybrid Genes as Well as Engineered Bacteria in Order to Produce Designed Biopolymer Beads

E. coli strain, plasmid, or oligonucleotide	genotype	source or reference
	Strains	
XL1-Blue	recA1, endA1, gyrA96, thi-1, hsdR17 (r^-_k, m^+_k), supE44, relA1, -, lac[F', proAB, lacI^q, lacZΔM15, Tn10(Tc^r)]	(*47*)
BL21(DE3)	F-, *ompT*, *hsdS*$_B$ (r$_B$- m$_B$-), *gal*, *dcm* (DE3)	Novagen
	Plasmids	
pBHR68	PHB biosynthesis operon from *Cupriavidus necator* in pBluescript SK$^-$	(*48*)
pHAS	pET-14b containing *Nde*I/*Bam*HI-inserted phaC gene from *C. necator*	(*49*)
pET-14b	Ap^r, T7 promoter	Novagen
pET-14b-ZZ(-)phaC	pET-14b containing *Xba*I/*Bam*HI fragment comprising gene *ZZ-phaC*	(*28*)
pCWE	pBluescript SK- derivative containing the PHA synthase gene from *C. necator*	(*16*)
pCWE-ZZ(-)phaC	pCWE derivative containing the ZZ domain-encoding *Nde*I fragment lacking the signal sequence-encoding region	(*28*)
pMCS69	pBBR1MCS derivative containing genes *pha*A and *pha*B of *C. necator* colinear to *lac* promoter	(*50*)
pCWE-GNphaC	pCWE derivative containing a *Xba*I/*Nde*I fusion of GN to 5' end of *phaC*	this study
pCWE-SNphaC	pCWE derivative containing a *Xba*I/*Nde*I fusion of SN to 5' end of *phaC*	this study
pCWE-3xGN-G$_5$-ZZ(-)phaC	pCWE derivative containing *Xba*I/*Sun*I fusion of 3GN to 5' end of ZZ(-)*phaC*	this study
pCWE- SN-G$_5$-ZZ(-)phaC	pCWE derivative containing *Xba*I/*Sun*I fusion of SN+ to 5' end of ZZ(-)*phaC*	this study
pET-14b-3xGN-G$_5$-ZZ(-)phaC	pET-14b derivative containing 2345 bp *Xba*I/*Bam*HI fragment of pCWE-3xGN-G$_5$-ZZ(-)phaC	this study
pET-14b-SN-G$_5$-ZZ(-)phaC	pET-14b derivative containing 2261 bp *Xba*I/*Bam*HI fragment of pCWE-SN-G$_5$-ZZ(-)phaC	this study

pET-14b-SNphaC	pET-14b derivative containing 1838 bp *Xba*I/*Bam*HI fragment of pCWE-SNphaC	this study
pET-14b-3xGN-G$_5$-ZZ(-)phaC / SN-G$_5$-ZZ(-)phaC	pET-14b-3xGN-G5-ZZ(-)phaC derivative containing *Bam*HI/*Blp*I SN-G$_5$-ZZ(-)phaC PCR product	this study
	Oligonucleotides	
GNI	5'-P-CTA GAA ATA AGG AGA TAT AGC AAT GCA TGG CAA AAC CCA GGC GAC CAG CGG CAC CAT TCA GAG CCA -3'	this study
GNII	5'-P-TAT GGC TCT GAA TGG TGC CGC TGG TCG CCT GGG TTT TGC CAT GCA TTG CTA TAT CTC CTT ATT T -3'	this study
SNI	5'-P-CTA GAA ATA AGG AGA TAT AGC AAT GAG CCC GCA TCC GCA TCC GCG CCA TCA TCA TAC CCA -3'	this study
SNII	5'-P-TAT GGG TAT GAT GAT GGC GCG GAT GCG GAT GCG GGC TCA TTG CTA TAT CTC CTT ATT T -3'	this study
SN+I	5'-P-CTA GAA ATA AGG AGA TAC TAG TAT GAG CCC GCA TCC GCA TCC GCG CCA TCA TCA TAC CCT CGA GC -3'	this study
SN+II	5'-P-GTA CGC TCG AGG GTA TGA TGA TGG CGC GGA TGC GGA TGC GGG CTC ATA CTA GTA TCT CCT TAT TT -3'	this study
3'PhaC-*Blp*I	5'- GCT CAG CGG TCA TGC CTT GGC TTT GAC GTA TCG CCC -3'	this study
*Bam*HI-SN+	5'- GGA TCC AAT AAG GAG ATA CTA GTA TGA GCC CGC ATC CGC AT -3'	this study

Isolation of Polymer Beads

The polymer beads were isolated by harvesting respective cells using centrifugation for 20 min at 6000 x g and 4˚C. The sediment was washed and suspended in 3 volumes of 50 mM phosphate buffer (pH 7.5). Cells were passed through a French Press two times at 16 kpsi. The cell lysate (0.75 ml) was loaded onto a glycerol gradient (88% and 44% (v/v) glycerol in phosphate buffer). After ultracentrifugation for 2.5 h at 100 000 x g and 10˚C, beads could be isolated from a white layer above the 88% glycerol layer. The PHA beads were washed with 10 volumes phosphate buffer (50 mM, pH 7.5) and centrifuged at 100 000 x g for 60 min at 10˚C. The sediment containing the PHA beads was suspended in phosphate buffer and stored at 4˚C.

Gold-Binding Assay

The gold-binding was carried out at 20°C in PKT buffer (10 mM KPO$_4$ (pH 7), 0.1 M KCl, 1% TritonX-100) with 250 μM HAuCl$_4$ and 200 μM sodium ascorbate according to Brown (*31*). For a 10 ml assay, a 1 mg/ml bead protein solution was used. The whole assay was incubated on a shaker until a pink color appeared, indicating the formation of colloidal gold. To let the gold bind to the peptides, the solution was further incubated. To wash the unbound gold out of the sample, the assay was centrifuged at 4000 x g for

15 min. The resulting sediment was suspended in 1 ml PKT buffer and loaded on a sucrose gradient (2.25 M – 0 M, 0.25 M increments, sucrose in PKT buffer). This gradient was undergoing ultracentrifugation for approximately 16 h at 100 000 x g. The resulting layer at 2 or 2.25 M sucrose, consisting of beads and gold, was washed with PKT buffer and centrifuged in an ultracentrifuge for 1 h at 100 000 x g. The sediment was used for TEM analysis and AFM measurements.

Silica-Binding Assays

The silica-binding assay was performed at room temperature according to Dickerson *et al.* (*34*). 1 mg/ml bead protein was dissolved in 50 mM potassium phosphate buffer, pH 7.5. The silica solution was freshly prepared by dissolving tetramethyl orthosilicate (TMOS, Sigma-Aldrich, St. Louis, MO) in 1 mM HCl to a final concentration of 1 M. The silica solution was then added to the protein solution so that the final concentration of TMOS in the silica-binding assay was 0.1 M. This mixture was then incubated on a shaker for 10 min and centrifuged for 5 min at 13 000 rpm. The sediment was washed three times with 50 mM potassium phosphate buffer, pH 7.5, to remove unbound silica. To investigate the bead density changes due to silica binding, the washed sediments were suspended in 50 mM potassium phosphate buffer, pH 7.5, and separated using a sucrose gradient (0 – 2.25 M sucrose in 0.25 M increments, sucrose in 50 mM potassium phosphate buffer, pH 7.5). These gradients underwent ultracentrifugation at 100 000 x g for 16 h at 10˚C. The resulting layers or sediments were washed in 50 mM potassium phosphate buffer, pH 7.5, and used for further investigations. In another silica binding assay, 0.5 mg/ml bead protein suspension was mixed with 0.003 g/ml SiO$_2$ (silica beads with a size range of 1-5 μm) in potassium phosphate buffer and incubated on a shaker at room temperature for 4 h. The mixture was separated by sucrose gradient ultracentrifugation. The protein concentration of resulting layers and sediments was determined by Bradford (*35*). To qualitatively assess the binding of the engineered polymer beads to silica beads, fluorescence microscopy was used. For this purpose, the polymer beads were selectively stained with the hydrophobic fluorescent dye Nile red.

ELISA

For the enzyme-linked immunosorbent assay (ELISA), the wells of microtiter plates were coated with 100 μl of a polymer bead suspension (normalized to protein concentration, in a range from 0.05 μg/μl to 0.4 x 10^{-3} μg/μl) and incubated overnight at 4°C. After being blocked with 3% (w/v) bovine serum albumin for 2 h, each well was incubated with 10 nm Gold-biotin (Nanocs Inc., New York, USA) for 2 h followed by 1 h incubation with streptavidin-horseradish peroxidase conjugate (streptavidin-HRP; Sigma-Aldrich, St. Louis, MO). After each step, the wells were washed several times with phosphate-buffered saline containing 0.05% Tween 20. As substrate, 100 μl of an o-phenylenediamine solution (Abbott Diagnostics, IL, USA) was added to each well, and after 15 min, the reaction was stopped by adding 100 μl of 1 N H_2SO_4. The amount of substrate conversion was measured at a wavelength of 490 nm using a microtiter plate reader.

Protein Analysis

Bead protein profiles were routinely analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described elsewhere (*36*). The gels were stained with Coomassie brilliant blue G250.

In order to identify proteins linked to the polymer beads, the bead proteins were separated by SDS-PAGE and protein bands of interest were cut off the gel. Proteins were subjected to MALDI-TOF mass spectrometry. Identification of tryptic peptides was performed by collision-induced dissociation tandem mass spectrometry and enabled identification of fusion proteins.

To identify N-terminally fused GEPIs, fusion proteins were subjected to N terminal sequencing using Edman degradation.

Microscopic Analysis

Atomic force microscopy imaging was performed on an Asylum Research MFP-3D instrument (Asylum Research, Santa Barbara, CA) in ac mode. All images were recorded in potassium phosphate buffer solution at a concentration of beads corresponding to 1-10 μg protein/ml. Higher bead concentrations were not used, as they resulted in scattering of the laser, which obscured the signal reflected from the cantilever and inhibited AFM operation. Approximately 100 μl of bead suspension was

placed on a gold-coated mica substrate shortly before imaging. AFM cantilevers were either silicon Ultrasharp NSG11 (NT–MDT Co, Moscow, Russia) with force constants in the range 5 – 25 N/m and a tip radius around 10 nm, or silicon nitride TR400PSA (Olympus Corporation, Japan) with a force constant around 0.08 N/m and a tip radius < 20nm. Images of 512 x 512 points were collected at line scan frequencies of 1 – 8 Hz.

For transmission electron microscopy (TEM) analysis, cells or beads were prepared as described previously (*26*). Sections were stained and assessed using a Philips CM10 TEM. To visualize the gold-binding, the beads were incubated with colloidal gold as described above in the gold-binding assay. Beads with bound colloidal gold or control beads not binding colloidal gold were then subjected to TEM sample preparation.

For elemental analysis, freeze-dried bead samples were mounted, using double- sided tape, on an aluminum specimen stub and then carbon-coated for EDS (energy dispersive spectrometry). Analysis was done using EDAX EDS system (EDAX, Mahwah, USA) in a FEI Quanta 200 SEM (scanning electron microscope; FEI, Eindhoven, The Netherlands). For imaging the gold in the samples, the BSED (back scattered electron detection) mode was used.

Table 2. Identified Peptide Fragments of Proteins Analyzed by MALDI-TOF/MS

Protein	Identified peptide fragments
SN-G_5-ZZ-PhaC	F34-R56, E37-R56, V175-R190, I217-R228, F269-R279, F300-R323, L324-R334, I349-R361, N362-K388, Y404-R417, H418-R432, N433-R453, G541-K567, L551-K567, E633-K652
3xGN-G_5-ZZ-PhaC	T5-K32 or F120-R142 or F62-R84, E65-R84 or E123-R142, N85-K106 or N143-K164, V174-R181, V203-R218, Q219-K244, I245-R256, Y257-K272, D260-K272, D260-R282, R283-R290, F284-R290, T291-R307, F297-R307, A308-K321, I326-R351, F328-R351, L352-R362, N367-R374, G375-R389, I377-R389, N390-K416, Y432-R445, H446-R460, N461-R481, I488-L517, Q553-A568, L569-N595, Y625-N633, P638-H660, I661-F680, G683-R700

Results

Design and Production of Bifunctional Gold/Antibody Binding Beads

The capability of bacteria to produce intracellular granules with a hydrophobic biopolymer core and proteins attached to its surface from various cheap carbon and energy sources, such as, e.g., glucose, was utilized in this study to produced tailor-made nano/micro-beads suitable for molecular biomimetics.

The bead-forming and coating protein PhaC was engineered to enable simultaneous bead surface display of the gold-binding peptide GBP1 (*31*) and the antibody (IgG) binding ZZ domain (*37*). This strategy harnesses nature's capability to covalently cross-link the PhaC protein to polymer inclusions, which can be isolated and maintained as spherical beads with defined surface binding functionality (*26*). In order to produce the various multi-functional fusion proteins in the bacterium *E. coli* that mediate production of gold-binding polymer beads, the respective hybrid genes were constructed and inserted into an overexpression vector under control of the strong T7 promoter. In general, bead formation was monitored by polyester accumulation analysis, fluorescence microscopy of Nile red stained cells, and TEM. A fusion protein with only one gold-binding peptide did not mediate production of beads showing gold-binding affinity (data not shown). However, the use of three gold-binding peptide repeats, a pentaglycine spacer region followed by the IgG-binding domain fused to PhaC, mediated formation of beads with specific gold and antibody binding properties. This fusion protein 3xGN-G_5-ZZ-PhaC at the bead surface was demonstrated by SDS-PAGE and was confirmed by MALDI-TOF/MS and N-terminal sequencing (Figure 2, Table 2). The SDS-PAGE analysis also showed an additional but less abundant 97 kDa protein, which was identified as copurified ATPase (Accession No. P0ABB9*)* derived from the production host *E. coli*.
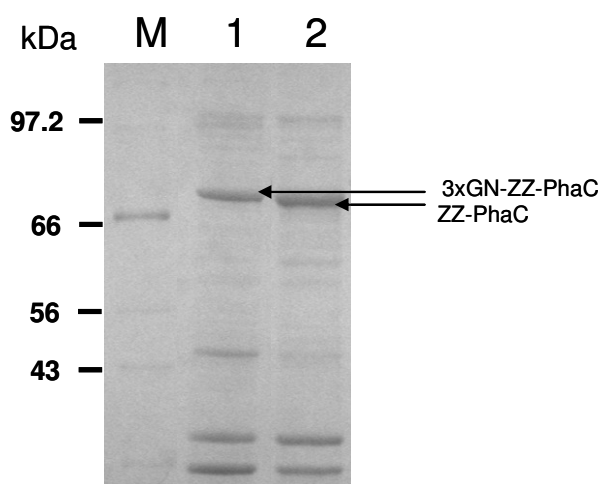

Figure 2. Protein profile of polymer beads displaying gold binding peptides. M, molecular mass standard (New England Biolabs, United Kingdom); Lane 1, polymer beads from *E. coli* BL21(DE3) (pET-14b-ZZ(-)phaC); lane 2, polymer beads from *E. coli* BL21(DE3) (pET-14b-3xGN-G_5-ZZ(-)phaC).

To quantitatively assess the gold-binding activity of these novel polymer beads, an enzyme-linked immunosorbent assay was established employing the binding of

biotinylated gold particles. Biotinylated gold particles bound to the beads were detected using streptavidin-HRP conjugate. The absorbance at 490 nm was increased at least 10-fold using the gold-binding peptide displaying beads when compared to the control beads not displaying the peptide (Figure 3A). Furthermore, elemental analysis and back-scattered electron density analysis was applied to confirm the presence of gold bound to the engineered beads (data not shown). The bead shape and size distribution was assessed by atomic force microscopy (data not shown).

The triple gold-binding peptide-ZZ-PhaC fusion protein additionally showed IgG binding activity due to a functional ZZ domain between the gold-binding peptides and PhaC. This activity was assessed by ELISA as previously described (*28*) (Figure 3B).
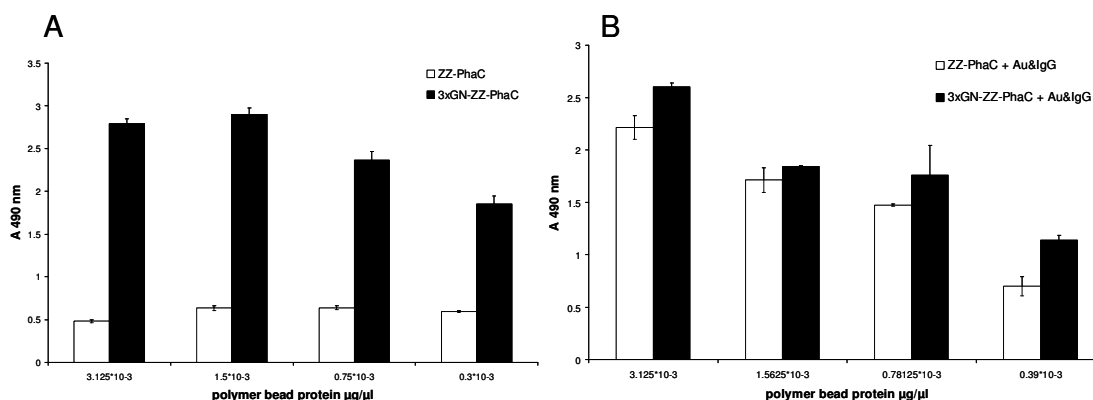

Figure 3. (A) Gold binding of various polymer beads assessed by ELISA. Polymer beads were isolated from *E. coli* BL21(DE3) harboring pET-14b-3xGN-G_5-ZZ(-)phaC (black columns) or pET-14b-ZZ(-)phaC (white columns). Streptavidin-HRP conjugate was used to detect bound 10 nm Biotin-Gold. Measurements were conducted in triplicates, the standard deviation is indicated. (B) Impact of gold-binding on IgG binding of various polymer beads assessed by ELISA. Polymer beads were isolated from *E. coli* BL21(DE3) harboring pET-14b-3xGN-G_5-ZZ(-)phaC and incubated with gold followed by an incubation with human IgG (3xGN-ZZ-PhaC + Au&IgG, black columns). As controls beads isolated from *E. coli* BL21(DE3) harboring pET-14b-ZZ(-)phaC were used and treated in the same way (gold incubation followed by human IgG incubation as ZZ-PhaC + Au&IgG, white columns). Goat polyclonal anti-human IgG-horseradish peroxidase conjugate was used for detection of bound human IgG. All measurements were conducted in triplicates, standard deviations are indicated.

The gold-binding was visualized by transmission electron microscopy (TEM) (Figure 4). To further study gold-binding and its impact on bead density, sucrose density gradients using ultracentrifugation were applied. The incubated samples as well as control samples were analyzed by sucrose gradient ultracentrifugation. Samples that were not incubated with colloidal gold formed a layer located at a density corresponding to < 2 M sucrose (*d* =1.33 g/ml). Control beads, PhaC(-)ZZ beads only displaying the IgG binding domain, when incubated with gold did not show a shift in

density. In contrast, beads displaying the gold-binding peptides migrated further in the density gradient and formed a layer corresponding to a density of < 2.25 M sucrose ($d =$ 1.4 g/ml) when incubated with gold (Figure 5). Overall, these data suggested that the engineered beads displayed both binding functionalities (Figure 6).
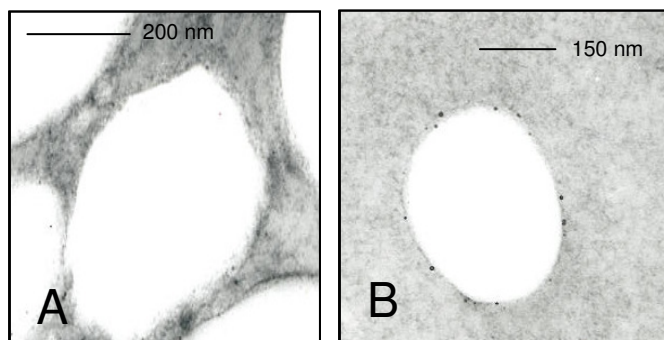

Figure 4. TEM images of polymer beads after incubation with colloidal gold. (A) Polymer beads displaying ZZ(-)phaC protein at the surface. (B) Polymer beads coated with the engineered protein 3xGN-G$_5$-ZZ(-)phaC mediating display of the gold-binding peptide. The pictures show slices through polymer beads embedded in BSA.
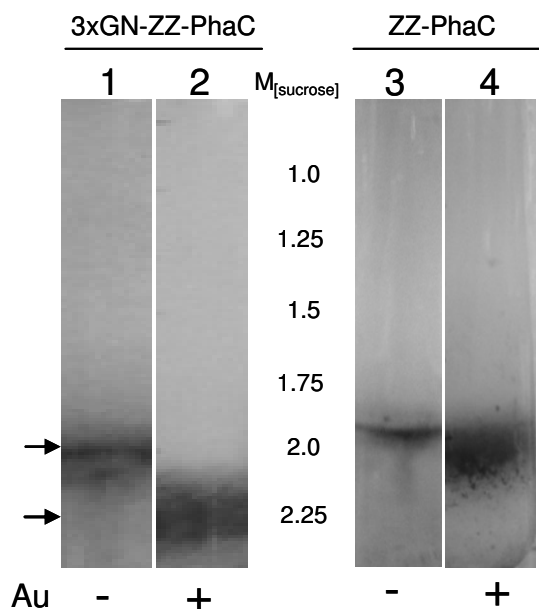

Figure 5. Gold-binding to polymer beads demonstrated by density dependent migration in a sucrose gradient using ultracentrifugation. Arrows indicate where the beads accumulated in the sucrose gradient after ultracentrifugation after incubation with 250 µM colloidal gold (+) and without (-). Lanes 1, 2, polymer beads from *E. coli* BL21(DE3)(pET-14b-3GN-G$_5$-ZZ(-)phaC); lanes 3, 4, polymer beads from *E. coli* BL21(DE3)(pET-14b-ZZ(-)phaC).
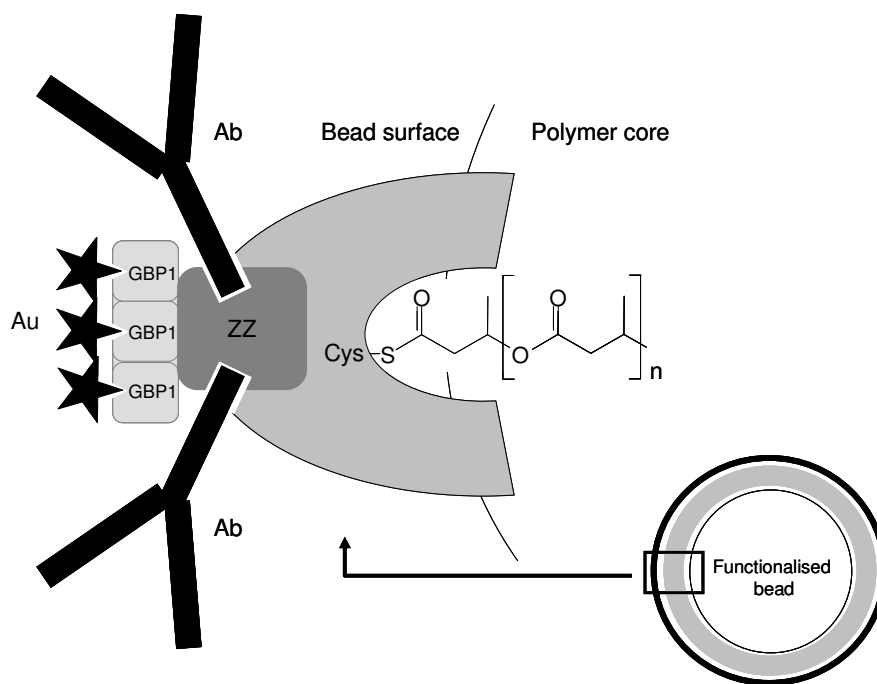
Figure 6. Scheme of the surface of a bifunctional gold/antibody binding polymer bead.

Design and Production of Bifunctional Silica/Antibody Binding Beads

As described above for the design of the gold/antibody-binding peptide displaying beads, hybrid genes were constructed to enable production of multifunctional fusion proteins mediating formation of silica/antibody-binding polymer beads. Hybrid genes were overexpressed in *E. coli* and the resulting beads were isolated and characterized (Figure 7). The fusion protein SN-G$_5$-ZZ-PhaC was confirmed by MALDI-TOF/MS and N terminal sequencing (Table 2). The SDS-PAGE analysis also showed an additional but less abundant 97 kDa protein, which was identified as copurified ATPase (accession no. P0ABB9) derived from the production host *E. coli*.

The ability of the respective beads displaying silica-binding peptides to bind silica was shown using two different methods assessing either binding of silica to the bead surface or binding of the engineered polymer beads to commercially available silica beads. Beads displaying silica-binding peptides and control beads were incubated with TMOS dissolved in HCl and then separated by sucrose density ultracentrifugation as described in the Experimental Procedures. Different migration behavior of the engineered and control beads was observed representing a change in bead density (Figure 8A). PhaC(-)ZZ beads formed a layer at the 2 M sucrose (d = 1.33 g/ml) independent on treatment with TMOS. The respective beads self-assembled using fusion protein SN-G$_5$-ZZ-PhaC
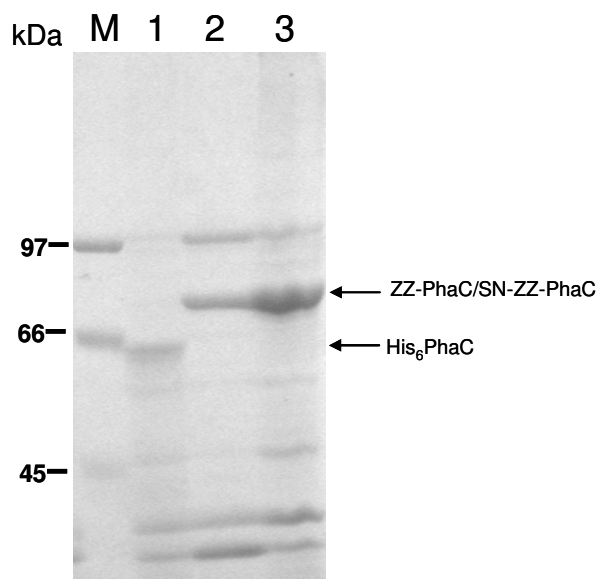
Figure 7. Protein profile of polymer beads displaying silica binding peptides. M, molecular mass standard (Amersham Biosciences, USA); lane 1, polymer beads from *E. coli* BL21(DE3) (pHAS); lane 2, polymer beads from *E. coli* BL21(DE3) (pET-14b-ZZ(-)phaC); lane 3, polymer beads from *E. coli* BL21(DE3) (pET-14b-SN-G_5-ZZ(-)phaC).

showed a density greater than the control beads and assembled at 2.25 M sucrose (d = 1.4 g/ml) after treatment with TMOS. These findings were additionally confirmed by elemental analysis showing silica only present in the engineered bead samples (data not shown). The direct fusion of the silica binding peptide to the N terminus of PhaC resulting in SN-PhaC did not result in an increased density. These beads always behaved like the control beads without binding peptide.

Commercially available silica beads (Silicon dioxide, 1-5 μm; Sigma-Aldrich, St. Louis, MO) were used as second method to qualitatively assess silica binding by the engineered beads. Fusion and control beads were incubated with silica beads and separated using sucrose density ultracentrifugation as described in the Experimental Procedures. Protein determination by Bradford (*35*) was used to quantify binding. The respective control beads PhaC(-)ZZ showed about 85% of the protein content at a density corresponding to the polymer beads not bound to the commercial silica beads, and about 15% was found at much greater density in the sediment colocalizing with the commercial silica beads. In contrast, about 70% of beads displaying the silica-binding peptide employing fusion protein SN-G_5-ZZ-PhaC colocalized with the commercial silica beads when separated in sucrose density ultracentrifugation (Figure 8B).
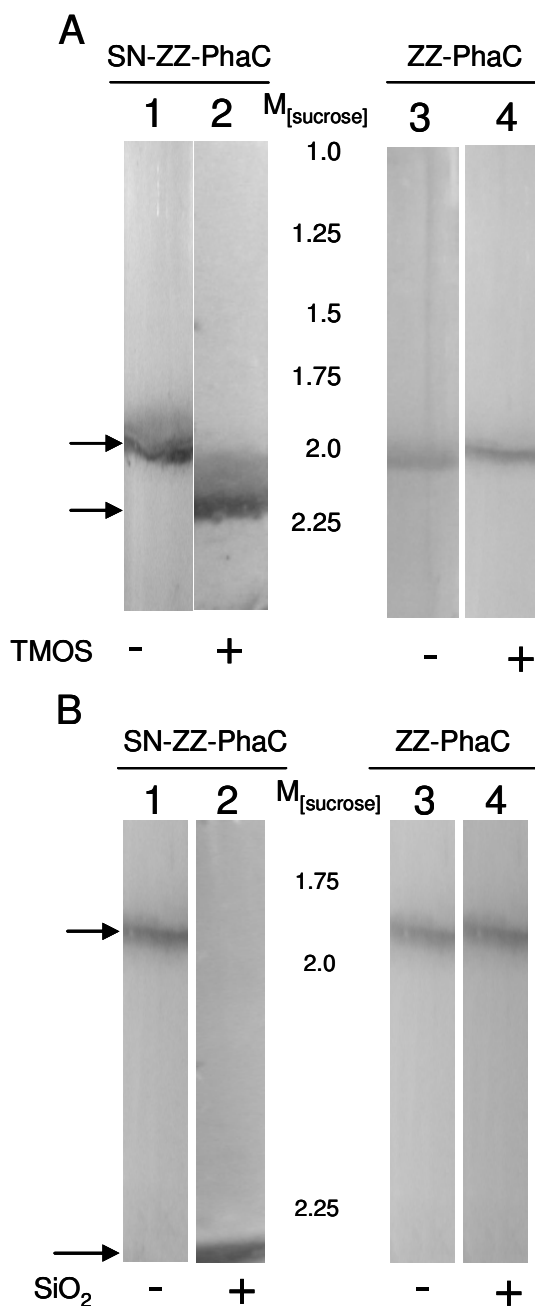
Figure 8. Silica-binding to polymer beads demonstrated by density dependent migration in a sucrose gradient using ultracentrifugation. (A) Images of tubes used for density assessment. Arrows indicate where the beads accumulated in the sucrose gradient after (+) and without (-) TMOS incubation. Lanes 1, 2, polymer beads from *E. coli* BL21(DE3) (pET-14b-SN-G$_5$-ZZ(-)phaC); lanes 3, 4, polymer beads from *E. coli* BL21(DE3) (pET-14b-ZZ(-)phaC). (B) Images of tubes used for density assessment. Arrows indicate the areas where the main part of the beads accumulated after SiO$_2$ binding (+) and without (-). Lanes 1, 2, polymer beads from *E. coli* BL21(DE3) (pET-14b-SN-G$_5$-ZZ(-)phaC); lanes 3, 4, polymer beads from *E. coli* BL21(DE3) (pET-14b-ZZ(-)phaC).

Design and Production of Multifunctional Gold/Silica/Antibody Binding Beads

As described for the design of the bifunctional beads, hybrid genes mediating production of multifunctional gold/silica/antibody-binding polymer beads were constructed and overexpressed in *E. coli*. Two different hybrid genes each encoding a fusion protein comprising bead forming and inorganic binding domain were co-expressed to mediate production of multifunctional beads (Figure 9). The fusion proteins were confirmed by MALDI-TOF/MS (data not shown).
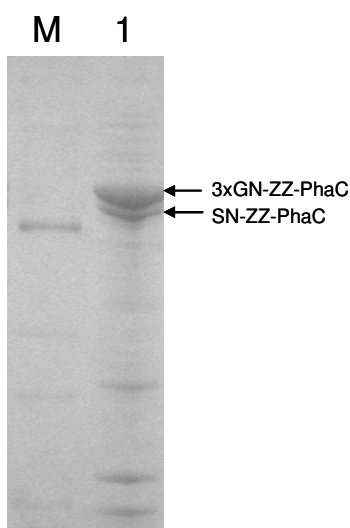

Figure 9. Protein profile of polymer beads displaying both binding activities (gold and silica). Lane M, molecular mass standard (New England Biolabs, United Kingdom); lane 1, polymer beads from *E. coli* BL21 (DE3) (pET-14b-3xGN-G_5- ZZ(-)phaC/SN-G_5-ZZ(-)phaC).

The gold and IgG binding ability of the respective beads was confirmed using ELISA as described above (data not shown). The silica-binding ability of the respective engineered beads was confirmed as described above (data not shown). To assess the multifunctionality, the respective engineered beads were incubated subsequently with colloidal gold and TMOS, and vice versa. After each incubation step, the samples were purified via sucrose density ultracentrifugation as described in the Experimental Procedures. As controls, the respective bifunctional polymer beads were incubated the same way as beads displaying the ZZ domain only. After incubation with colloidal gold followed by TMOS incubation, PhaC(-)ZZ beads accumulated in an area corresponding to 2 M sucrose (d = 1.33 g/ml), whereas bifunctional gold/antibody binding beads formed a layer located at a density of 2.25 M sucrose (d = 1.4 g/ml). Multifunctional gold/silica/antibody binding beads

treated the same way migrated even further and formed a layer corresponding to a density of 2.5 M sucrose ($d = 1.45$ g/ml; Figure 10).
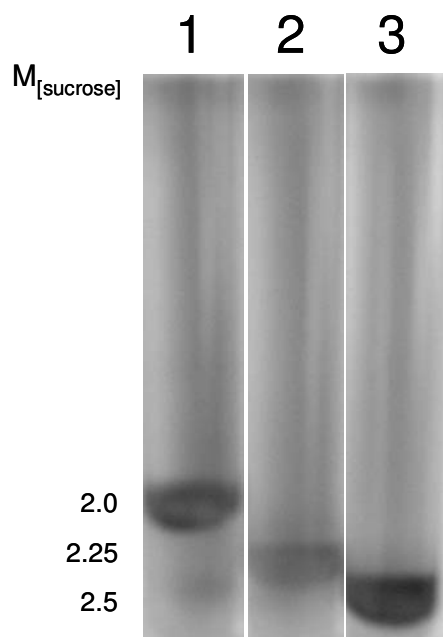

Figure 10. Different polymer beads were incubated with gold and silica and purified via ultracentrifugation of a sucrose gradient. Lane 1, pET-14b-ZZ(-)phaC; lane 2, pET-14b-3xGN-G$_5$-ZZ(-)phaC; lane 3, pET-14b-3xGN-G$_5$-ZZ(-)phaC/SN-G$_5$-ZZ(-)phaC.

Discussion

In this study, it was shown that microorganisms can be genetically modified to produce polymer inclusions that perform as beads displaying binding sites for antibodies and various inorganics at the same time. Thus, the bioengineering of polymer inclusions provides a valid molecular tool to generate novel hybrid materials that combine biological and inorganic agents suitable for numerous potential biomedical applications (*2-5*). Different hybrid genes were constructed (Figure 11) and successfully overexpressed, and the engineered protein functions could be displayed at the respective bead surfaces (Figures 2, 7 and 9). The gold/antibody binding polymer beads displaying the triple gold-binding peptide GBP1 (*38, 39*) showed strong gold binding in ELISA, which was established as a tool to quantitatively assess the gold binding. Control beads that did not display this special binding peptide showed no significant gold binding (Figure 3A). These findings were confirmed by density-based migration behavior of the different beads in a sucrose gradient. Only beads displaying the gold-

binding peptide showed an increased density after incubation with colloidal gold. TEM assessment of the polymer beads indicated specific gold-binding to respectively engineered beads (Figure 4). However, the density of bound colloidal gold was rather low which could be due to the isolation procedure applied to beads after binding the colloidal gold as well as the TEM sample preparation method. Additionally, no interference of both displayed binding domains – gold-binding and IgG-binding – was found. Moreover, it could be demonstrated that polymer beads engineered to bind gold and antibodies bind as much IgG as polymer beads displaying only the antibody binding domain (Figure 3B).
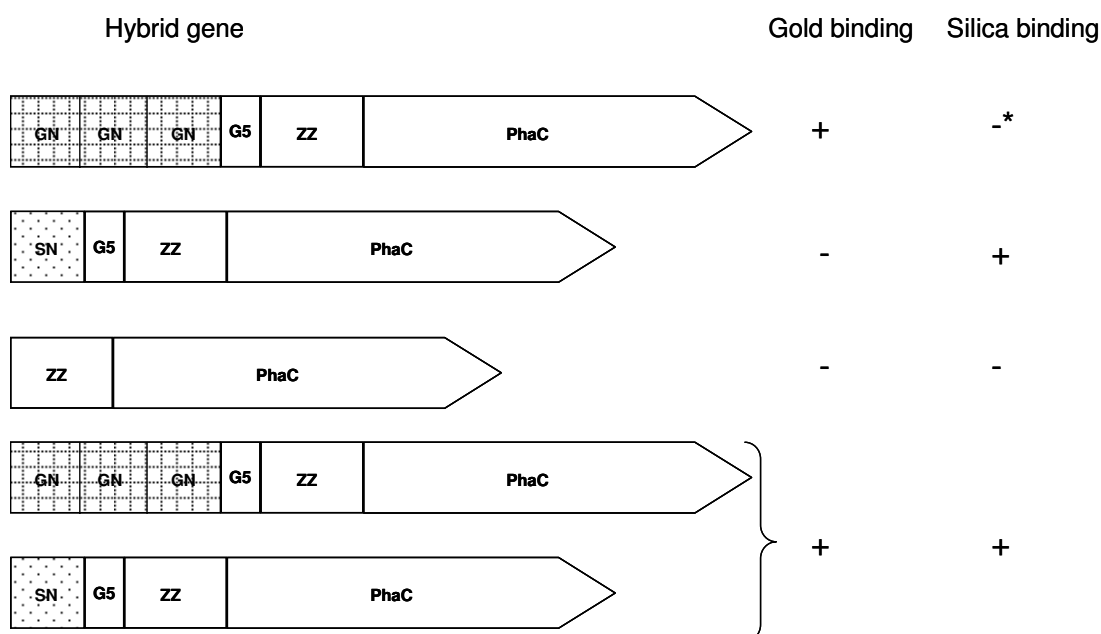

Figure 11. Schematic overview of hybrid genes used in this study. The bracket combining the last two constructs illustrates that both genes are located on the same plasmid. Gold binding ability was assessed as described above and the silica-binding ability was assessed using TMOS precipitation as described above. Binding is indicated by +, non-binding by -, -* indicates a slightly increased unspecific silica binding compared to the control.

To test the possibility that the polymer beads can be in general designed to bind any inorganic, the protein bead surface was designed to bind silica. The binding of silica, obtained as precipitate from an aqueous TMOS solution, increased the density of the respective engineered beads, whereas the control beads without engineered binding sites showed no increased density (Figure 8A,B). Further confirmation of the successful functional displaying of the silica-binding peptide was obtained by assessing the binding of engineered SN-G$_5$-ZZ-PhaC and PhaC(-)ZZ control beads to commercial

silica particles. Control beads showed only a minor background reaction compared to the binding ability of the engineered beads (Figure 8 C,D). A direct fusion of the silica binding peptide to the N terminus of PhaC did not result in functional silica binding polymer beads. Therefore, the exposure of the binding peptide – gained by employing the IgG binding ZZ domain as spacer – seems to be required for functionality. Elemental analysis using energy dispersive spectrometry proved the existence of silica in samples that were expected to precipitate silica out of TMOS, e.g., silica-binding peptide displaying beads. Silica could not be detected in control samples treated the same way. Due to the displayed ZZ domain, the engineered beads were additionally able to bind IgG as well as the control beads only displaying the ZZ domain. In contrast to the gold-binding, it was not possible to obtain clear evidence that IgG-binding and silica-binding do not interfere with each other. This is due to the lack of an ELISA protocol for silica binding to the beads.

The multifunctional beads designed in this study were not only able to bind IgG antibodies plus gold and/or silica, but were also able to specifically bind all three compounds. However, it was shown that a deposition of silica at the bead surface impaired binding of gold, whereas beads with gold attached to their surface showed no weaker silica deposition as in the absence of gold. These data suggested that the silica deposition could block the binding sites for gold at the bead surface. Additionally, multifunctional beads incubated with gold and silica showed a higher density in sucrose gradients than observed for bifunctional beads. This indicates a possible binding of the silica to the already bound gold particles. Overall, this study clearly demonstrated that bacterial polymeric inclusions can be engineered as biomimetic tools to specifically and simultaneously bind biological and inorganic compounds, which could enable applications of these beads in diagnostic medicine as well as biotechnology. Numerous particles composed of various materials such as organic polymers, semi-conductors, lipids, carbon- or silica-material have been produced and proposed for various biomedical applications (*40-46*). In contrast to the one-step manufacturing process described in this study for the production of new polymer beads, all other particles requiring functionalization were obtained by a process comprising at least two steps such as, e.g., bead production and chemical cross-linking of a functionality.

This biomimetic approach offers a tool to produce beads with covalently cross-linked functionalities in one-step. These shell-core bio-beads with specific binding properties could, for example, be applicable in medical bioimaging procedures in which antibody-

based targeting of contrast agents is of special interest. In a previous study, it was demonstrated that a scFv immobilized at the surface of these beads showed a 75-fold increase in binding affinity when compared with the respective soluble scFv (*26*). Thus, the engineered beads could provide a very sensitive diagnostic tool. Moreover, the module like composition of these small bio-beads offers versatile targeting of different tissues by simply incubating the beads with the relevant targeting antibody. Due to the displayed antibody binding functionality, an antibody of choice or even a combination of antibodies could be easily combined with bound gold to generate a contrast agent for bioimaging applications.

Acknowledgement

References

(1)   Addinall, S. G., Bi, E., and Lutkenhaus, J. (1996) FtsZ ring formation in fts mutants. *J. Bacteriol. 178*, 3877-84.

(2)   Karhanek, M., Kemp, J. T., Pourmand, N., Davis, R. W., and Webb, C. D. (2005) Single DNA molecule detection using nanopipettes and nanoparticles. *Nano Let.t 5*, 403-7.

(3)   Jiang, W., Papa, E., Fischer, H., Mardyani, S., and Chan, W. C. (2004) Semiconductor quantum dots as contrast agents for whole animal imaging. *Trends Biotechnol. 22*, 607-9.

(4)   Panyam, J., and Labhasetwar, V. (2003) Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug Deliveru Rev. 55*, 329-47.

(5)   Soukka, T., Harma, H., Paukkunen, J., and Lovgren, T. (2001) Utilization of kinetically enhanced monovalent binding affinity by immunoassays based on multivalent nanoparticle-antibody bioconjugates. *Anal. Chem. 73*, 2254-60.

(6)   Mao, C., Solis, D. J., Reiss, B. D., Kottmann, S. T., Sweeney, R. Y., Hayhurst, A., Georgiou, G., Iverson, B., and Belcher, A. M. (2004) Virus-based toolkit for the directed synthesis of magnetic and semiconducting nanowires. *Science 303*, 213-7.

(7)   Tamerler, C., and Sarikaya, M. (2007) Molecular biomimetics: utilizing nature's molecular ways in practical engineering. *Acta Biomater. 3*, 289-99.

(8)   Sarikaya, M., Tamerler, C., Jen, A. K., Schulten, K., and Baneyx, F. (2003) Molecular biomimetics: nanotechnology through biology. *Nat. Mater. 2*, 577-85.

(9)   Zin, M. T., Ma, H., Sarikaya, M., and Jen, A. K. (2005) Assembly of gold nanoparticles using genetically engineered polypeptides. *Small 1*, 698-702.

(10) Oren, E. E., Tamerler, C., and Sarikaya, M. (2005) Metal recognition of septapeptides via polypod molecular architecture. *Nano Lett. 5*, 415-9.

(11) Thai, C. K., Dai, H., Sastry, M. S., Sarikaya, M., Schwartz, D. T., and Baneyx, F. (2004) Identification and characterization of Cu(2)O- and ZnO-binding polypeptides by *Escherichia coli* cell surface display: toward an understanding of metal oxide binding. *Biotechnol. Bioeng. 87*, 129-37.

(12) Rehm, B. H. A. (2006) Biopolyester particles produced by microbes or using polyester synthases: Self-assembly and potential applications.
In Microbial Bionanotechnology: Biological self-assembly systems and biopolymer-based nanostructures, ed. Bernd H. A. Rehm, pp 1-34.

(13) Rehm, B. H. A. (2006) Genetics and Biochemistry of Polyhydroxyalkanoate Granule Self-assembly: The Key Role of Polyester Synthases. *Biotechnol. Lett. 28*, 207-13.

(14) Rehm, B. H. A. (2007) Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. *Curr. Issues Mol. Biol. 9*, 41-62.

(15) Peters, V., Becher, D., and Rehm, B. H. A. (2007) The inherent property of polyhydroxyalkanoate synthase to form spherical PHA granules at the cell poles: the core region is required for polar localization. *J. Biotechnol. 132*, 238-45.

(16) Peters, V., and Rehm, B. H. A. (2005) In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. *FEMS Microbiol. Lett. 248*, 93-100.

(17) Rehm, B. H. A. (2003) Polyester synthases: natural catalysts for plastics. *Biochem. J. 376*, 15-33.

(18) Peters, V., and Rehm, B. H. A. (2006) In vivo enzyme immobilization by use of engineered polyhydroxyalkanoate synthase. *Appl. Environ. Microbiol. 72*, 1777-83.

(19) Hezayen, F. F., Steinbuchel, A., and Rehm, B. H. A. (2002) Biochemical and enzymological properties of the polyhydroxybutyrate synthase from the extremely halophilic archaeon strain 56. *Arch. Biochem. Biophys. 403*, 284-91.

(20) Potter, M., Muller, H., Reinecke, F., Wieczorek, R., Fricke, F., Bowien, B., Friedrich, B., and Steinbuchel, A. (2004) The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha. Microbiology 150*, 2301-11.

(21) Jurasek, L., and Marchessault, R. H. (2004) Polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* cells: a computer simulation. *Appl. Microbiol. Biotechnol. 64*, 611-7.

(22) York, G. M., Stubbe, J., and Sinskey, A. J. (2001) New insight into the role of the PhaP phasin of *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate. *J. Bacteriol. 183*, 2394-7.

(23) Baeckstroem, T. B., Brockelbank, J. A., and Rehm, B. H. A. (2007) Recombinant *Escherichia coli* produces tailor-made biopolyester granules for applications in fluorescence activated cell sorting: Functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein. *BMC Biotechnol. 7*, 3.

(24) Banki, M. R., Gerngross, T. U., and Wood, D. W. (2005) Novel and economical purification of recombinant proteins: intein-mediated protein purification using in vivo polyhydroxybutyrate (PHB) matrix association. *Protein Sci. 14*, 1387-95.

(25) Barnard, G. C., McCool, J. D., Wood, D. W., and Gerngross, T. U. (2005) Integrated recombinant protein expression and purification platform based on *Ralstonia eutropha. Appl. Environ. Microbiol. 71*, 5735-42.

(26) Grage, K., and Rehm, B. H. A. (2008) In Vivo Production of scFv-Displaying Biopolymer Beads Using a Self-Assembly-Promoting Fusion Partner. *Bioconjugate Chem. 19*, 254-62.

(27) Peters, V., and Rehm, B. H. A. (2008) Protein engineering of streptavidin for in vivo assembly of streptavidin beads. *J. Biotechnol. 134*, 266-74.

(28) Brockelbank, J. A., Peters, V., and Rehm, B. H. A. (2006) Recombinant *Escherichia coli* strain produces a ZZ domain displaying biopolyester granules

suitable for immunoglobulin G purification. *Appl. Environ. Microbiol. 72*, 7394-7.

(29) Sharma, P., Brown, S., Walter, G., Santra, S., and Moudgil, B. (2006) Nanoparticles for bioimaging. *Adv. Colloid Interface Sci. 123-126*, 471-85.

(30) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual* Cold Spring Harbor Lab. Press, Plainview, NY.

(31) Brown, S. (1997) Metal-recognition by repeating polypeptides. *Nat. Biotechnol. 15*, 269-272.

(32) Brown, S., Sarikaya, M., and Johnson, E. (2000) A genetic analysis of crystal growth. *J. Mol. Biol. 299*, 725-735.

(33) Naik, R. R., Brott, L. L., Clarson, S. J., and Stone, M. O. (2002) Silica-precipitating peptides isolated from a combinatorial phage display peptide library. *J. Nanosci. Nanotechnol. 2*, 95-100.

(34) Dickerson, M. B., Naik, R. R., Sarosi, P. M., Agarwal, G., Stone, M. O., and Sandhage, K. H. (2005) Ceramic nanoparticle assemblies with tailored shapes and tailored chemistries via biosculpting and shape-preserving inorganic conversion. *J. Nanosci. Nanotechnol. 5*, 63-67.

(35) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248-54.

(36) Laemmli, U. K. (1970) Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4. *Nature 227*, 680-685.

(37) Lowenadler, B., Jansson, B., Paleus, S., Holmgren, E., Nilsson, B., Moks, T., Palm, G., Josephson, S., Philipson, L., and Uhlen, M. (1987) A gene fusion system for generating antibodies against short peptides. *Gene 58*, 87-97.

(38) Tamerler, C., Duman, M., Oren, E. E., Gungormus, M., Xiong, X., Kacar, T., Parviz, B. A., and Sarikaya, M. (2006) Materials specificity and directed assembly of a gold-binding peptide. *Small 2*, 1372-8.

(39) Tamerler, C., Oren, E. E., Duman, M., Venkatasubramanian, E., and Sarikaya, M. (2006) Adsorption kinetics of an engineered gold binding Peptide by surface plasmon resonance spectroscopy and a quartz crystal microbalance. *Langmuir 22*, 7712-8.

(40) Chenevier, P., Veyret, B., Roux, D., and Henry-Toulme, N. (2000) Interaction of cationic colloids at the surface of J774 cells: a kinetic analysis. *Biophys. J. 79*, 1298-309.

(41) Nayak, S., and Lyon, L. A. (2005) Soft nanotechnology with soft nanoparticles. *Angew. Chem., Int. Ed. Engl. 44*, 7686-708.

(42) Schultz, D. A. (2003) Plasmon resonant particles for biological detection. *Curr. Opin. Biotechnol. 14*, 13-22.

(43) Alivisatos, A. P., Johnsson, K. P., Peng, X., Wilson, T. E., Loweth, C. J., Bruchez, M. P., Jr., and Schultz, P. G. (1996) Organization of 'nanocrystal molecules' using DNA. *Nature 382*, 609-11.

(44) Venkatesan, N., Yoshimitsu, J., Ito, Y., Shibata, N., and Takada, K. (2005) Liquid filled nanoparticles as a drug delivery tool for protein therapeutics. *Biomaterials 26*, 7154-63.

(45) Burns, A., Sengupta, P., Zedayko, T., Baird, B., and Wiesner, U. (2006) Core/Shell fluorescent silica nanoparticles for chemical sensing: towards single-particle laboratories. *Small 2*, 723-6.

(46) Burns, A., Ow, H., and Wiesner, U. (2006) Fluorescent core-shell silica nanoparticles: towards "Lab on a Particle" architectures for nanobiotechnology. *Chem. Soc. Rev. 35*, 1028-42.

(47) Bullock, W. O., Fernandez, J. M., and Short, J. M. (1987) XL1-BLUE - A High-Efficiency Plasmid Transforming RecA Escherichia-Coli Strain with Beta-Galactosidase Selection. *Biotechniques 5*, 376-&.

(48) Spiekermann, P., Rehm, B. H. A., Kalscheuer, R., Baumeister, D., and Steinbuchel, A. (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch. Microbiol. 171*, 73-80.

(49) Yuan, W., Jia, Y., Tian, J. M., Snell, K. D., Muh, U., Sinskey, A. J., Lambalot, R. H., Walsh, C. T., and Stubbe, J. (2001) Class I and III polyhydroxyalkanoate synthases from *Ralstonia eutropha* and *Allochromatium vinosum*: Characterization and substrate specificity studies. *Arch. Biochem. Biophys. 394*, 87-98.

(50) Hoffmann, N., Amara, A. A., Beermann, B. B., Q, Q. S., Hinz, H. J., and Rehm, B. H. A. (2002) Biochemical characterization of the *Pseudomonas putida* 3-

hydroxyacyl ACP : CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. *J. Biol. Chem. 277*, 42926-42936.

Chapter IV

Engineering of bacterial polyester inclusions towards the display of ten lysine residues and potential applications

Anika C. Jahns, Verena Peters and Bernd H. A. Rehm*

Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

* Corresponding author: Bernd H. A. Rehm, phone: +64 6 350 5515 ext 7890; fax: +64 6 350 2267; e-mail: b.rehm@massey.ac.nz

Engineering of bacterial polyester inclusions towards the display of ten lysine residues and potential applications

Anika C. Jahns, Verena Peters and Bernd H. A. Rehm

Abstract

Lysine residues are positively charged and are known to enable silica binding. Silica is a very versatile element with various applications in biotechnology and biomedicine. In this study, *Escherichia coli* was engineered to produce polylysine displaying polyester beads, using one of the most dominant surface proteins, the phasins, which are naturally attached to these hydrophobic beads, as anchor. These shell-core beads were composed of a polyhydroxybutyrate core surrounded by hybrid gene encoded phasin-polylysine fusion proteins at a high density as was assessed by gas chromatography-mass spectroscopy and tryptic peptide fingerprinting using MALDI-TOF/MS, respectively. The resulting particles performed successfully in different silica binding assays and can be applied in processes where silica-coated biological particles are of special interest, e.g. DNA binding and delivery. The beads can also be used for applications where the display of a positive surface charge is advantageous.

Introduction

The element silicon is the second most abundant element in earth's crust and ubiquitous throughout biological systems. Silica exhibits diverse and extraordinarily designed shapes and structures in Nature from diatom skeletons to sponge spicules. The proteins directing silica synthesis in biological systems have been studied extensively. Silaffins extracted from the cell wall of diatom *Cylindrotheca fusiformis* catalyze precipitation of silica from aqueous solutions of orthosilicic acid [1-4]. Silicatein extracted from the sponge spicules of *Tethya aurantia* [5-7] is another good example for these proteins. A second approach is to use polyaminoacids to induce silica synthesis. Polyaminoacids were proposed to bring the silicic acid monomers and oligomers closer to each other by electrostatic attractions, which may facilitate condensation to produce silica [8]. Using

biomimetics offers a tool to synthesize inorganic materials under mild conditions, favourable in the biological world.

In this study microbial polyester inclusions were designed as biological matrix structure [9, 10]. These polyesters are composed of (*R*)-3-hydroxy fatty acids and serve as energy and carbon storage material [11]. The storage polyesters are deposited in the cytoplasm of bacteria or archaea as water-insoluble spherical inclusions and can contribute up to 80% (w/w) of the cellular dry weight matter of cells. These polyester inclusions have recently been considered as nano/micro-beads, whereby the protein coat can be genetically designed, applying protein engineering to display specific functionalities [12-14]. A dominant coat protein, the so-called phasin, is hydrophobically interacting with the polyester core. These proteins show a rather low molecular-weight of about 25 kDa and represent a class of non-catalytic proteins comprising a hydrophobic domain, which associates with the polyester core, and a hydrophilic domain exposed to the cytoplasm of the cell [15]. Phasin proteins are synthesized in very large quantities under storage conditions, representing as much as 5 % of the total protein [16].

These main structural proteins can be engineered to display various functional proteins at the polymer surface (Figure 1) and the modified polymer inclusions can be stably maintained outside the cell as beads [14, 17].
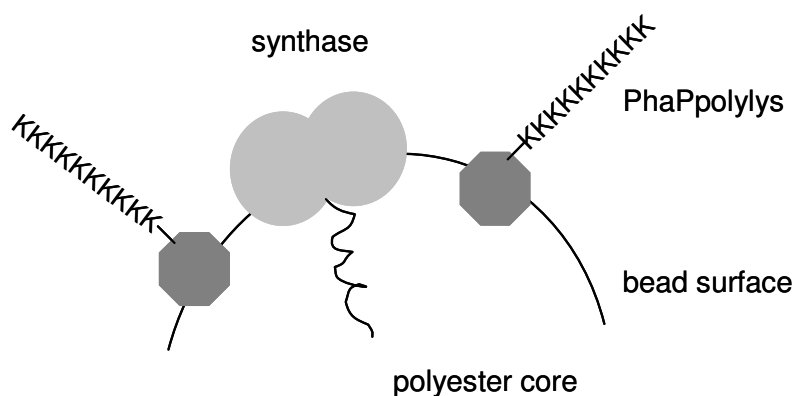

Figure 1. Scheme of the polyester bead surface displaying polylysine residues using engineered PhaP protein as anchor.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* BL21(DE3) was grown at 25°C. When required, ampicillin 75 µg/ml and chloramphenicol 50 µg/ml were added. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Isolation, analysis and manipulation of DNA

General cloning procedures and isolation of DNA were performed as described elsewhere [18]. DNA primers, deoxynucleoside triphosphate, T4 DNA ligase and Pfx polymerase were purchased from Invitrogen ™ (CA, USA). DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic sequencer.

The sequence of *phaP* was PCR amplified from genomic DNA from *Cupriavidus necator*, using the oligonucleotides 5'-PhaP-Xba/SD and 3'-PhaP-Xho/Spe/Xba (Table 1). The PCR product was ligated into the *Xba*I hydrolysed plasmid pHAS, resulting in plasmid pHAS+phaPwt. A C terminal fusion of 10 lysines to the hydrophobically bead binding protein PhaP was designed to enable silica-binding by the respective beads. Therefore, the oligonucleotides polylysine-adaptor and polylysine-adaptor rev (Table 1) were hybridised and inserted into the *Xho*I/*Spe*I sites of plasmid pHAS+phaPwt, resulting in plasmid pHAS+phaPpolylys.

The plasmid pETC [19] was hydrolysed with *Xba*I/*Nde*I. The oligonucleotides polylysine XbaI and polylysine NdeI were hybridised and inserted into the respectively prepared plasmid, resulting in plasmid pET-14b polylys-PhaC. The plasmid pET-14b ZZ(-)phaC [20] was hydrolysed with the restriction enzymes *Spe*I and *Sun*I. The oligonucleotides polylysine SpeI and polylysine SunI were hybridised and ligated into the prepared plasmid, resulting in plasmid pET-14b polylys-ZZ-PhaC.

Competent *E. coli* BL21(DE3) cells containing the plasmid pMCS69 were transformed with the newly constructed plasmids, respectively. Plasmid pMCS69 encodes the *β*-ketothiolase and acetyl-CoA reductase from *C. necator*. Together with the PHA synthase, encoded on pHAS or pET-14b derivatives, the activity of all three enzymes leads to polyester inclusion formation.

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain	Genotype	source or reference
Strains		
Escherichia coli		
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17(r-$_k$, m+$_k$), supE44, relA1, -, lac[F', proAB, lacI^q, lacZΔM15, Tn10(Tc^r)]*	[37]
BL21(DE3)	F-, *ompT, hsdS*$_B$ (r$_B$- m$_B$-), *gal, dcm* (DE3)	Novagen
Cupriavidus necator H16	Wildtype	DSMZ 428
Plasmids		
pETC	pET-14b derivative, coding for *phaC*wt under T7 promoter control	[19]
pET-14b-ZZ(-)phaC	pET-14b containing *Xba*I/*Bam*HI fragment comprising gene *ZZ-phaC*	[20]
pHAS	pET-14b containing *Nde*I/*Bam*HI-inserted *phaC* gene from *C. necator*	[38]
pHAS+phaPwt	pHAS plasmid containing *phaP* gene of *C. necator*	This study
pHAS+phaPpolylys	pHAS+phaP derivative containing polylysine fusion 3' to *phaP*	This study
pMCS69	pBBR1MCS derivative containing genes *phaA* and *phaB* of *C. necator* colinear to lac promoter	[39]
Oligonucleotides		
5'-PhaP-Xba/SD	5'-GGA AAT CTA GAA ATA AGG AGA TAT AGA TAT GAT CCT CAC CCC GGA ACA AGT TGC AGC AGC GC-3'	This study
3'-PhaP-Xho/Spe/Xba	5'-AAA AAT CTA GAT CAA CTA GTT CAC TCG AGG GCA GCC GTC GTC TTC TTT GCC GTG GCC GTA C-3'	This study
polylysine-adaptor	5'-P-TCG AGA AGA AGA AAA AGA AAA AAA AGA AGA AGA AGT AGA-3'	This study
polylysine-adaptor rev	5'-P-CTA GTC TAC TTC TTC TTC TTT TTT TTC TTT TTC TTC TTC-3'	This study
polylysine XbaI	5'-P-CTA GAA ATA AGG AGA TAC TAG TAT GAA GAA GAA AAA GAA AAA AAA GAA GAA GAA GCA-3'	This study
polylysine NdeI	5'-P-TAT GCT TCT TCT TCT TTT TTT TCT TTT TCT TCT TCA TAC TAG TAT CTC CTT ATT T-3'	This study
polylysine SpeI	5'-P-CTA GTA TGA AGA GAA AAA GAA AAA AGA AGA AGA AGC-3'	This study
polylysine SunI	5'-P-GTA CGC TTC TTC TTC TTT TTT TTC TTT TTC TTC TTC ATA-3'	This study

GC/MS

The polyester content of isolated cells was determined by gas chromatography/mass spectrometry [21].

Isolation of polyester inclusions

Polyester beads were isolated from *E. coli* cultures as described previously [22].

Silica-binding assay

The silica-binding assay was modified as described elsewhere [23] and as described previously [22].

To investigate the different migration behaviour due to bound silica, the washed pellets were suspended in 50 mM potassium phosphate buffer, pH 7.5 and loaded on a sucrose gradient (0 – 2.25 M sucrose in 0.25 M increments, sucrose in 50 mM potassium phosphate buffer, pH 7.5). These gradients were undergoing ultracentrifugation at 100 000 x g for 16 h.

Commercial silica beads in the range of 1-5 µm were used as well to assess the silica binding ability of the respective polyester beads. 0.5 mg/ml bead protein suspension was mixed with 0.003 g/ml SiO_2 in potassium phosphate buffer and incubated on a shaker at room temperature for 4 h. The mixture was loaded on a sucrose gradient and undergoing ultracentrifugation for 16 h at 10°C. The protein concentration of resulting layers and pellets was determined by Bradford [24].

SDS page

Polyester bead protein samples were analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described elsewhere [25]. The gels were stained with Coomassie brilliant blue G250.

MALDI-TOF mass spectrometry

Protein bands of interest were cut off the SDS polyacrylamide gel and analyzed by tryptic peptide fingerprinting applying collision induced dissociation tandem mass spectrometry.

DNA-binding

Beads incubated with TMOS and washed with KPO_4 were used in a DNA-binding assay. 35 µg of PhaPwt and PhaPpolylys (TMOS) beads were incubated with 675 ng DNA in 25 µl QG buffer (QIAquick® Gel Extraction Kit, QIAGEN, Hilden, Germany) for 1 h (RT, rocking). After washing with 50 µl PE buffer (QIAquick® Gel Extraction Kit, QIAGEN, Hilden, Germany), bound DNA was eluted in 25 µl EB buffer (10 mM Tris-HCl, pH 8.5). The assays were incubated on a rocker for 1 h, before the beads were sedimented by centrifugation. All supernatants were assessed for their DNA

content, using a Qubit^{TM} Fluorometer (Invitrogen^{TM}, CA, USA) and DNA gel electrophoresis.

Results

Analysis of the polylysine fusion protein displaying beads

The polyester inclusion coating protein PhaP, which specifically and hydrophobically binds to already formed polyester beads, was engineered to comprise 10 lysine residues at its C terminus. The respective hybrid gene and the polyester synthase were overexpressed under T7 promoter control in *E. coli* BL21 harbouring plasmid pMCS69, which mediated polyester synthesis and inclusion formation. The resulting beads were isolated and analyzed (Figure 2). All constructs were confirmed by DNA sequencing, the polyester content was measured by GC/MS and the fusion protein PhaPpolylys was confirmed by MALDI-TOF/MS (Table 2).
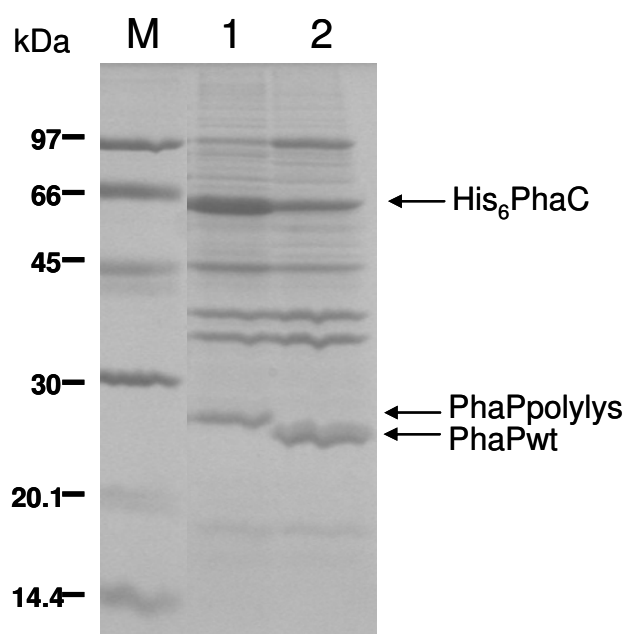

Figure 2. Protein profile of polyester beads displaying polylysines. M, molecular mass standard (Amersham Biosciences, USA); lane 1, polyester beads from *E. coli* BL21(DE3)(pHAS+phaPpolylys); lane 2, polyester beads from *E. coli* BL21(DE3)(pHAS+phaPwt).

Table 2. Identified peptide fragments of protein analyzed by MALDI-TOF/MS

Protein	Identified peptide fragments
PhaPpolylys	M1-K13, A14-K25, L33-K42, T43-K53, D60-K78, H85-K99, N111-K121, S136-K156, Q154-K172, A173-R182

Silica-binding

The ability of the respective polyester beads to bind silica was shown in two different assays by binding silica to the bead surface and by incubating polyester beads with commercially available silica beads. In the first approach, engineered beads and control beads were incubated with TMOS dissolved in HCl as described and then loaded on a sucrose gradient. After ultracentrifugation for 16 h different migration behaviour, i.e. different density due to silica binding, of engineered and control beads was observed. Plain polyester beads in which production was mediated by plasmid pHAS+phaPwt, formed a layer at a sucrose concentration of 2 M ($d = 1.33$ g/ml), and at 2.25 M ($d = 1.4$ g/ml) after TMOS incubation. PhaPpolylys beads showed a layer at 2 M sucrose when non-incubated with TMOS and formed a pellet below the 2.25 M sucrose after TMOS incubation (Figure 3A).
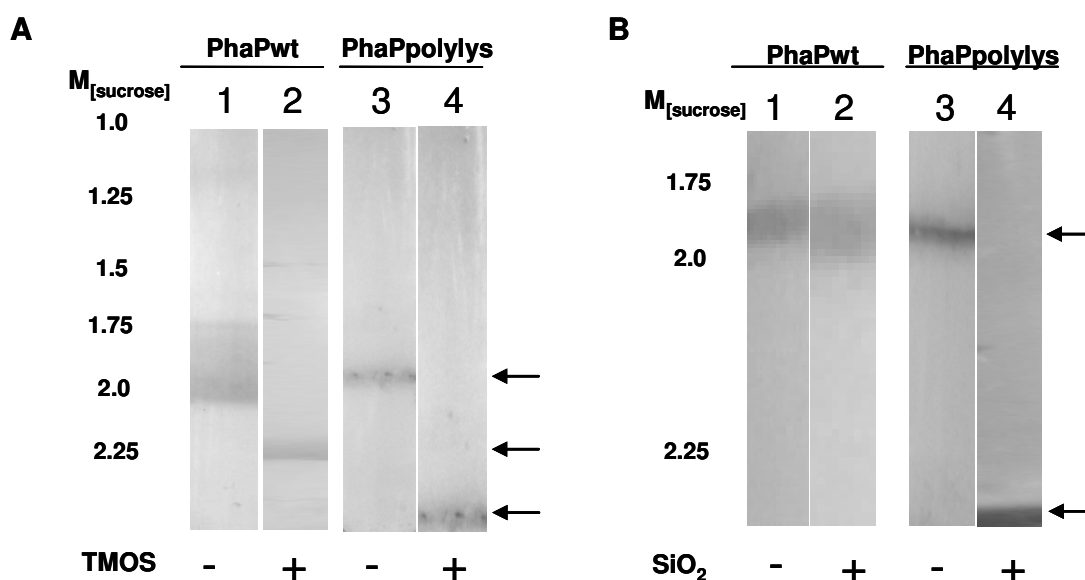

Figure 3. Density dependent migration in a sucrose gradient using ultracentrifugation. **A**. Images of tubes used for density assessment of the distribution of polyester beads in sucrose gradients after (indicated as +) and without (indicated as -) TMOS incubation. Lanes 1, 2, polyester beads from *E. coli* BL21(DE3)(pHAS+phaPwt); lanes 3, 4, polyester beads from *E. coli* BL21(DE3)(pHAS+ phaPpolylys). **B**. Images of tubes used for density assessment of the distribution of polyester beads after SiO$_2$ binding (indicated with +) and without (indicated with -). Lanes 1, 2, polyester beads from *E. coli* BL21(DE3)(pHAS+phaPwt); lanes 3, 4, polyester beads from *E. coli* BL21(DE3) (pHAS+ phaPpolylys).

Commercially available silica beads (Silicon dioxide, 1-5 µm; Sigma-Aldrich) were used for the second approach. PhaPpolylys and control beads were incubated with these silica beads as described and loaded onto a sucrose gradient. Protein determination by Bradford was used to quantify binding of the protein coated polyester beads. The

respective control beads coated with the wild-type phasin only showed about 75% of the protein content in the respective polyester bead layer in the sucrose gradient whereas only 25% were found bound to the silica beads in the sediment. The PhaPpolylys beads showed only about 30% of the protein content in the polyester bead layer, while most of the protein was found attached to the silica beads in the sediment (Figure 3B).

DNA-binding

To demonstrate the applicability of silica coated beads, DNA-binding to the silicated bead surface was investigated. After successful binding of silica, respectively prepared beads were assessed for their DNA-binding ability. PhaPwt and PhaPpolylys beads with bound silica (TMOS assay) were incubated with DNA as described. 250.5 ng DNA (54%) were eluted from control beads, 547 ng DNA (72%) were eluted from PhaPpolylys beads, app. 1.3 times more compared to PhaPwt beads. Fluorometric quantification as well as agarose gel electrophoresis was employed to assess the DNA-binding.
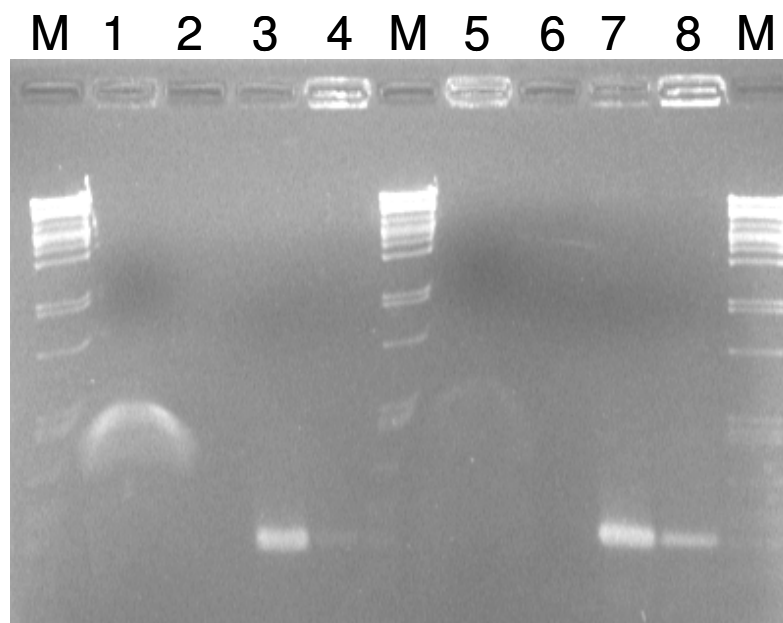

Figure 4. DNA-binding and elution from silica coated bio-beads. Beads were incubated with DNA, all supernatants and bead fractions were subjected to agarose gel electrophoresis. M, molecular weight standard (*PstI* digested lambda-DNA); lane 1, beads coated with PhaPwt, supernatant after DNA binding; lane 2, beads coated with PhaPwt, supernatant after wash; lane 3, beads coated with PhaPwt, supernatant after elution; lane 4, beads coated with PhaPwt, bead pellet in TE buffer; lane 5, beads coated with PhaPpolylys, supernatant after DNA binding; lane 6, beads coated with PhaPpolylys, supernatant after wash; lane 7, beads coated with PhaPpolylys, supernatant after elution; lane 8, beads coated with PhaPpolylys, bead pellet in TE buffer.

Analysis of polyester beads displaying the polylysine polypeptide via synthase fusion

The synthase protein PhaC, which is covalently bound to the polyester beads, was also employed for the display of the polypeptide. Ten lysine residues were fused to the N terminus of PhaC. In order to further expose the polypeptide, the ZZ-PhaC protein was engineered by N terminal fusion of 10 lysine residues. The hybrid genes were overexpressed under T7 promoter control in *E. coli* BL21 containing plasmid pMCS69. Polyester beads were isolated and analyzed. The presence of the fusion protein on the polyester bead surface could be demonstrated by SDS-PAGE and MALDI-TOF/MS (data not shown). However, none of the polyester beads harbouring either of the polylys-synthase fusion protein showed activity regarding silica precipitation (data not shown).

Discussion

The chemical synthesis of silica-based materials like resins and catalysts requires extremes of temperature, pressure and pH. In contrast, biosilification processes take place at ambient temperatures and pressures, but still producing a wide diversity of nanostructures [26-29]. Most of the proteins directing silica synthesis in biological systems are rich in basic and hydroxyl amino acids [30]. Moreover, it could be shown that synthetic peptides and polyamines can also be used to mimic biosilification reactions [8, 31-34].

In this study it was demonstrated that a polyamine can be functionally displayed at the surface of a biological derived polyester bead when genetically fused to a structural surface protein. SDS-PAGE analysis clearly showed the presence of the fusion protein PhaPpolylys at the bead surface (Figure 2). Binding experiments proved the activity. Polylysine can precipitate silica out of an aqueous solution within minutes [35]. In a silica binding assay and following sucrose density gradient characterization it could be shown that this activity of the polylysine peptide was not decreased by fusing it to the protein anchor PhaP (Figure 3A). Control beads derived from pHAS+phaPwt showed some background reaction which might be due to proteins attached to the bead surface. In comparison, engineered beads displaying lysine residues showed a more intense shift in density after incubation with TMOS solution due a significantly enhanced silica

binding. Furthermore, the polypeptide enabled the engineered beads to bind specifically to commercially silicon dioxide particles (Figure 3B). In this assay only little background reaction of the control beads could be observed, emphasizing the role of polylysines for silica precipitation and binding.

As proof-of-principle, silica coated beads were applied for specific DNA binding and elution. This assay needs some adjustments for future biomedical and biotechnological applications, as the background reaction was relatively high and not all DNA was eluted from the beads. Nevertheless, polylysine displaying beads incubated with TMOS were able to bind and elute 1.3 times more DNA than control beads (Figure 4). This clearly demonstrates the potential of biological, silicated beads.

It was tried to also use the covalently granule bound polyester synthase protein for the display of the polypeptide. Fusion proteins mediating polyester bead formation and localized at high density at the bead surface could be obtained but none of those beads mediated silica precipitation (data not shown). These results suggested a distinct surface exposure of the polylysines when displayed attached at the C terminus of the phasin protein, whereas a fusion to the N terminus of the synthase protein might not be fully displayed at the bead surface or might allow conformational changes of the N terminal region interfering with its accessibility. Previous studies clearly demonstrated that various N-terminal fusion to the synthase resulted in displayed and accessible functionalities, but it had not been attempted to only display ten lysine residues which strongly enhance the surface charge suitable for binding of an inorganic compound [12].

Future applications for silica-bound polyester beads could be in DNA purification processes while harnessing the adsorption of DNA molecules to silica. Another potential application could be the use of these silica-coated beads in drug delivery. In a recent study it has been shown that the uptake of encapsulated therapeutic agents might be facilitated by displayed polylysine residues [36]. Moreover, non-silicated beads could be used for cross-linking experiments due to the display of lysine residues which ε–amino group provides a target for various chemical reactions.

Acknowledgement

References

[1] N. Kröger; R. Deutzmann; C. Bergsdorf; M. Sumper, "Species-specific polyamines from diatoms control silica morphology," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 97, no 26, pp 14133-14138, 2000.

[2] N. Kröger; R. Deutzmann; M. Sumper, "Polycationic peptides from diatom biosilica that direct silica nanosphere formation," *Science*, Vol. 286, no 5442, pp 1129-1132, 1999.

[3] N. Kröger; R. Deutzmann; M. Sumper, "Silica-precipitating peptides from diatoms - The chemical structure of silaffin-1A from *Cylindrotheca fusiformis*," *Journal of Biological Chemistry*, Vol. 276, no 28, pp 26066-26070, 2001.

[4] N. Kröger; M. Sumper, Biomineralization: From Biology to Biotechnology and Medical Application, In Bauerlein, E. Ed. Wiley-VCH: Weinheim, Germany, (2000); pp 151-170.

[5] K. Shimizu; J. Cha; G. D. Stucky; D. E. Morse, "Silicatein alpha: Cathepsin L-like protein in sponge biosilica," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 95, no 11, pp 6234-6238, 1998.

[6] Y. Zhou; K. Shimizu; J. N. Cha; G. D. Stucky; D. E. Morse, "Efficient catalysis of polysiloxane synthesis by silicatein alpha requires specific hydroxy and imidazole functionalities," *Angewandte Chemie-International Edition*, Vol. 38, no 6, pp 780-782, 1999.

[7] J. N. Cha; K. Shimizu; Y. Zhou; S. C. Christiansen; B. F. Chmelka; G. D. Stucky; D. E. Morse, "Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones *in vitro*," *Proceedings of the National*

Academy of Sciences of the United States of America, Vol. 96, no 2, pp 361-365, 1999.

[8]   T. Coradin; O. Durupthy; J. Livage, "Interactions of amino-containing peptides with sodium silicate and colloidal silica: A biomimetic approach of silicification," *Langmuir*, Vol. 18, no 6, pp 2331-2336, 2002.

[9]   B. H. A. Rehm, "Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: The key role of polyester synthases," *Biotechnology Letters*, Vol. 28, no 4, pp 207-213, 2006.

[10]   B. H. A. Rehm, "Biopolyester particles produced by microbes or using the polyester synthases: Self-assembly and potential applications", In *Microbial Biotechnology: Biological self-assembly systems and biopolymer-based nanostructures*, Rehm, B. H. A. Ed. Horizon Bioscience: Norfolk, UK, pp 1-34, 2006.

[11]   H. G. Schlegel; H. Kaltwasser; G. Gottschalk, "[A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies.]," *Archiv für Mikrobiologie*, Vol. 38, pp 209-222, 1961.

[12]   B. H. A. Rehm, "Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles," *Current Issues in Molecular Biology*, Vol. 9, no 1, pp 41-62, 2007.

[13]   K. Grage; B. H. A. Rehm, "*In Vivo* Production of scFv-Displaying Biopolymer Beads Using a Self-Assembly-Promoting Fusion Partner," *Bioconjugate Chemistry*, Vol. 19, no 1, pp 254-262, 2008.

[14]   T. B. Bäckström; J. A. Brockelbank; B. H. A. Rehm, "Recombinant *Escherichia coli* produces tailor-made biopolyester granules for applications in fluorescence activated cell sorting: Functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein," *BMC Biotechnology*, Vol. 7, no 1, p 3, 2007.

[15]   S. Z. Hanley; D. J. C. Pappin; D. Rahman; A. J. White; K. M. Elborough; A. R. Slabas, "Re-evaluation of the primary structure of *Ralstonia eutropha* phasin and implications for polyhydroxyalkanoic acid granule binding," *FEBS Letters*, Vol. 447, no 1, pp 99-105, 1999.

[16]   A. Steinbüchel; K. Aerts; W. Babel; C. Follner; M. Liebergesell; M. H. Madkour; F. Mayer; U. Pieper-Fürst; A. Pries; H. E. Valentin; e. al., "Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions," *Canadian Journal of Microbiology*, Vol. 41 Suppl 1, pp 94-105, 1995.

[17] J. A. Atwood; B. H. A. Rehm, "Protein engineering towards biotechnological production of bifunctional polyester beads," *Biotechnology Letters*, Vol. 31, no 1, pp 131-137, 2009.

[18] J. Sambrook; E. F. Fritsch; T. Maniatis, *Molecular cloning: a laboratory manual*, Cold Spring Harbor Lab. Press: Plainview, NY, 1989.

[19] V. Peters; B. H. A. Rehm, "Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads," *Journal of Biotechnology*, Vol. 134, pp 266-274, 2008.

[20] J. A. Brockelbank; V. Peters; B. H. Rehm, "Recombinant *Escherichia coli* strain produces a ZZ domain displaying biopolyester granules suitable for immunoglobulin G purification," *Applied and Environmental Microbiology*, Vol. 72, no 11, pp 7394-7397, 2006.

[21] H. Brandl; R. A. Gross; R. W. Lenz; R. C. Fuller, "*Pseudomonas oleovorans* as a Source of Poly(beta-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters," *Applied and Environmental Microbiology*, Vol. 54, no 8, pp 1977-1982, 1988.

[22] A. C. Jahns; R. G. Haverkamp; B. H. A. Rehm, "Multifunctional inorganic-binding beads self-assembled inside engineered bacteria," *Bioconjugate Chemistry*, Vol. 19, no 10, pp 2072-2080, 2008.

[23] M. B. Dickerson; R. R. Naik; P. M. Sarosi; G. Agarwal; M. O. Stone; K. H. Sandhage, "Ceramic nanoparticle assemblies with tailored shapes and tailored chemistries via biosculpting and shape-preserving inorganic conversion," *Journal of Nanoscience and Nanotechnology*, Vol. 5, no 1, pp 63-67, 2005.

[24] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochememistry*, Vol. 72, pp 248-254., 1976.

[25] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, Vol. 227, no 5259, pp 680-685, 1970.

[26] S. Mann, "Molecular tectonics in biomineralization and biomimetic materials chemistry," *Nature*, Vol. 365, no 6446, pp 499-505, 1993.

[27] S. Mann; G. A. Ozin, "Synthesis of inorganic materials with complex form," *Nature*, Vol. 382, no 6589, pp 313-318, 1996.

[28] S. Mann; J. Webb; R. J. P. Williams, *Biomineralization: Chemical and Biochemical Perspectives*, Mann, S.; Webb, J.; Williams, R. J. P. Eds.; VCH: Weinheim, Germany, 1989.

[29] S. Oliver; A. Kuperman; N. Coombs; A. Lough; G. A. Ozin, "Lamellar aluminophosphates with surface patterns that mimic diatom and radiolarian microskeletons," *Nature*, Vol. 378, no 6552, pp 47-50, 1995.

[30] C. Tamerler; M. Sarikaya, "Molecular Biomimetics: Linking Polypeptides to Inorganic Structures", In *Microbial Biotechnology: Biological Self-assembly Systems and Biopolymer-based Nanostructures*, Rehm, B. H. A. Ed. Horizon Bioscience: Norfolk, UK, 2006.

[31] R. R. Naik; L. L. Brott; S. J. Clarson; M. O. Stone, "Silica-precipitating peptides isolated from a combinatorial phage display peptide library," *Journal of Nanoscience and Nanotechnology*, Vol. 2, no 1, pp 95-100, 2002.

[32] S. V. Patwardhan; N. Mukherjee; M. Steinitz-Kannan; S. J. Clarson, "Bioinspired synthesis of new silica structures," *Chemical Communications*, no 10, pp 1122-1123, 2003.

[33] M. R. Knecht; S. L. Sewell; D. W. Wright, "Size control of dendrimer-templated silica," *Langmuir*, Vol. 21, no 5, pp 2058-2061, 2005.

[34] T. Coradin; J. Livage, "Effect of some amino acids and peptides on silicic acid polymerization," *Colloids and Surfaces, B: Biointerfaces*, Vol. 21, no 4, pp 329-336, 2001.

[35] M. M. Tomczak; D. D. Glawe; L. F. Drummy; C. G. Lawrence; M. O. Stone; C. C. Perry; D. J. Pochan; T. J. Deming; R. R. Naik, "Polypeptide-templated synthesis of hexagonal silica platelets," *Journal of the American Chemical Society*, Vol. 127, no 36, pp 12577-12582, 2005.

[36] J. K. Vasir; V. Labhasetwar, "Quantification of the force of nanoparticle-cell membrane interactions and its influence on intracellular trafficking of nanoparticles," *Biomaterials*, Vol. 29, no 31, pp 4244-4252, 2008.

[37] W. O. Bullock; J. M. Fernandez; J. M. Short, "XL1-BLUE - A high-efficiency plasmid transforming RecA *Escherichia coli* strain with beta-galactosidase selection," *BioTechniques*, Vol. 5, no 4, p 376, 1987.

[38] W. Yuan; Y. Jia; J. M. Tian; K. D. Snell; U. Muh; A. J. Sinskey; R. H. Lambalot; C. T. Walsh; J. Stubbe, "Class I and III polyhydroxyalkanoate synthases from *Ralstonia eutropha* and *Allochromatium vinosum*: Characterization and substrate

specificity studies," *Archives of Biochemistry and Biophysics*, Vol. 394, no 1, pp 87-98, 2001.

[39] N. Hoffmann; A. A. Amara; B. B. Beermann; Q. Qi; H. J. Hinz; B. H. A. Rehm, "Biochemical characterization of the *Pseudomonas putida* 3-hydroxyacyl ACP:CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis," *Journal of Biological Chemistry*, Vol. 277, no 45, pp 42926-42936, 2002.

Chapter V

Conclusions

Conclusions

One focus of this thesis was to investigate whether the PHA synthase PhaC from *Ralstonia eutropha* can be genetically engineered at its C terminus. Previous studies demonstrated the importance of the C terminus for activity of the protein and therefore no modification was ever attempted. The results described in Chapter II clearly show that PhaC can be employed as a multiple anchor by modifying the N and C terminus of this protein with different functionalities. In accordance with previous publications, the findings reported in Chapter II emphasise the importance of the hydrophobic environment around the synthase C terminus to maintain functionality. The ability to produce bio-beads displaying multiple functionalities originating from the one essential polymerising surface protein broadens the applicability of these beads as a greater variety of desired functions can be displayed.

Furthermore, the research of this thesis demonstrated the applicability of functionalised bio-beads for molecular biomimetics approaches. The bio-bead surface was engineered to promote the specific binding of antibodies and inorganic materials, e.g. gold or silica. The capacity of the bead surface was not limited to the binding of only one type of inorganic material; two different inorganics could be bound at the same time. The results presented in Chapter III clearly demonstrate the binding of gold and silica to respectively engineered multi-functional beads. Genetically engineered peptides for inorganics (GEPIs) are available for a wide range of inorganic materials, and therefore the bead surface could be engineered to bind any desired combination of materials.

In a different biomimetic approach, the bead surface associated protein PhaP was engineered to display 10 lysine residues. Bio-beads displaying these positively charged amino acid residues are very versatile in their applicability. As a proof-of-principle, silica binding to these engineered beads was investigated and employed to detect the correct display of the lysine residues.

Outlook

Particles of a wide range of biopolyester materials are currently being investigated regarding their applicability especially in biomedicine and biotechnology. So far, the production costs of these bio-beads are high compared to well-established petrochemically derived products. Therefore, only high-value applications would justify the high production costs. Also, size and uniformity of the particles as well as consistency and reproducibility of the performance are demanded from every product. Although there are some insights into size control of PHB beads, further studies are necessary to provide an optimal product. This includes different growth media as well as different incubation temperatures and incubation times. The outcome of these studies might also provide further indication about how to reduce the production costs while maintaining or even improving the quality of the product. Using a two-plasmid system for the recombinant production of PHB bio-beads implements a greater performance variety and therefore a higher possibility of inconsistency and non-reproducibility. On the other hand, results reported so far and described in this thesis clearly show that this system is simple but allows a cost-effective one-step production and the resulting bio-beads show little variability in their performance. As there are only three enzymes required for the production of these bio-beads, namely PhaA, PhaB and PhaC, all three genes encoding these enzymes could be transcribed from one plasmid. This would decrease the selection pressure, as only one antibiotic would be required and thereby also reducing production costs. In a future experiment pursuing this strategy all three genes could be encoded together on a pET-14b derivative. The synthase gene would be controlled by the strong T7 promoter, whereas the two genes for *phaA* and *phaB* would be controlled by the lac promoter.

Biomedical applications of PHA derived biopolyester beads are widely emerging and might be the only field where high production costs are permitted at the moment. After first reports of experiments in mouse (Yao *et al*., 2008), it could well be imagined that specially designed biopolyester beads will find applications not only in diagnostics but in drug delivery as well. Beads loaded with the drug of interest, encapsulated into the hydrophobic core of the beads, could be delivered by antibody-targeting using the well characterized antibody binding capability (Brockelbank *et al*., 2006). For monitoring purposes, bioimaging using gold, as an inorganic contrast agent, bound via genetically

engineered peptides for inorganics to the particle surface (Jahns *et al.*, 2008), can be performed.

For bio-medical applications of the bio-beads an effective method for pyrogen removal would be necessary, as currently used methods are often harsh and might sometimes cause inactivation of the desired functionality. Mainly, recombinant *E. coli* cells are used as production hosts and consequently the beads are prone to contamination with host-specific lipopolysaccharides. However, *E. coli* has been approved by the U.S. Food and Drug Administration (FDA) for the production of biopharmaceuticals. Current research has shown that the lactic acid bacterium *Lactococcus lactis* can be genetically engineered to produce PHB beads (Mifune *et al.*, 2009). *L. lactis* is classified as a food-grade, generally-regarded-as-safe organism and does not produce lipopolysaccharides. Therefore, this organism would be an ideal host for the production of bio-beads with medical applications. This technology is relatively new and still needs further investigation and improvement, and would not be favourable for the production of beads that are not required to be pyrogen-free. *E. coli* is a well established production host, the genome is well studied and a wide variety of techniques are available for easy manipulation and engineering. To date, beads purified from *E. coli* as well as *L. lactis* are contaminated with respective host proteins that bind unspecifically to the bead surface. The amount of these contaminating proteins can be reduced by strong overproduction of the desired fusion protein which results, presumably, in less space available on the bead surface for the unspecific binding proteins. High salt and mild detergent washing can also improve the purity of the beads but it has to be ensured that the bead itself and the protein of interest displayed at the bead surface remain active.

More research concerning translational fusions to the C terminus of the PHA synthase is necessary in order to exploit the full capacity of the bead surface. The work of this thesis has provided a general insight into the possibilities, but for oriented tailoring of special products, a better and faster cloning strategy might be desirable. Additionally, determination of the crystal structure of PhaC might help to establish potential interactions and interferences between fusion partners. Further insights into the structure and folding mechanism might help to explain why some of the fusion proteins described in Chapter II remained inactive, especially the phenomena of the synthase and GFP protein separated by the designed linker sequence. So far, all attempts of enrichment of the synthase protein resulted in insoluble protein that cannot be used for

crystallization assays. New strategies are currently being pursued, e.g. addition of a solubility tag or expression of a shorter version of the protein, to enhance the solubility of the enriched protein.

References

Brockelbank, J. A.; Peters, V.; Rehm, B. H. A. *Appl. Environ. Microbiol.*, **2006,** *72*, 7394-7397.

Jahns, A. C.; Haverkamp, R. G.; Rehm, B. H. A. *Bioconj. Chem.*, **2008,** *19*, 2072-2080.

Mifune, J.; Grage, K.; Rehm, B. H. A. *Appl. Environ. Microbiol*., **2009**, 75, 4668-4675.

Yao, Y.-C.; Zhan, X.-Y.; Zhang, J.; Zou, X.-H.; Wang, Z.-H.; Xiong, Y.-C.; Chen, J.; Chen, G.-Q. *Biomaterials*, **2008,** *29*, 4823-4830

Chapter VI

Appendix

Appendix

SDS-PAGE

Most of the SDS-PAGE pictures shown in this thesis are composite images. In some cases samples were run on the same gel but separated by additional, not shown, lanes. In any case, all samples were run under the same conditions, even when run on different gels.

An average of 30 µg of total protein was loaded per lane. The protein concentration was determined following the method developed by M. M. Bradford (1976).

The SDS-PAGE pictures always show, in addition to the fusion proteins of interest, contaminating protein bands. These proteins bind in an unspecific manner to the isolated beads, most likely during the isolation process. Some of these contaminating proteins were identified by MALDI-TOF/MS. The protein with an average molecular mass of 97 kDa was identified as *E. coli* derived ATPase. The smaller proteins with molecular masses between 30 and 40 kDa were identified as *E. coli* elongation factor TU (42 kDa), outer membrane protein OmpA (35 kDa) and β-Lactamase TEM precursor (32 kDa).

Construction of plasmids encoding C-terminal synthase fusions (Chapter II)

For better understanding and traceability, a schematic of the cloning strategy leading to the different plasmids employed in this study is shown in Figures 1 and 2.

Construction of plasmids used for biomimetic studies (Chapter III)

Figures 3 and 4 show schematics of the cloning strategy employed that led to the plasmids encoding fusion proteins that are able to bind inorganics.
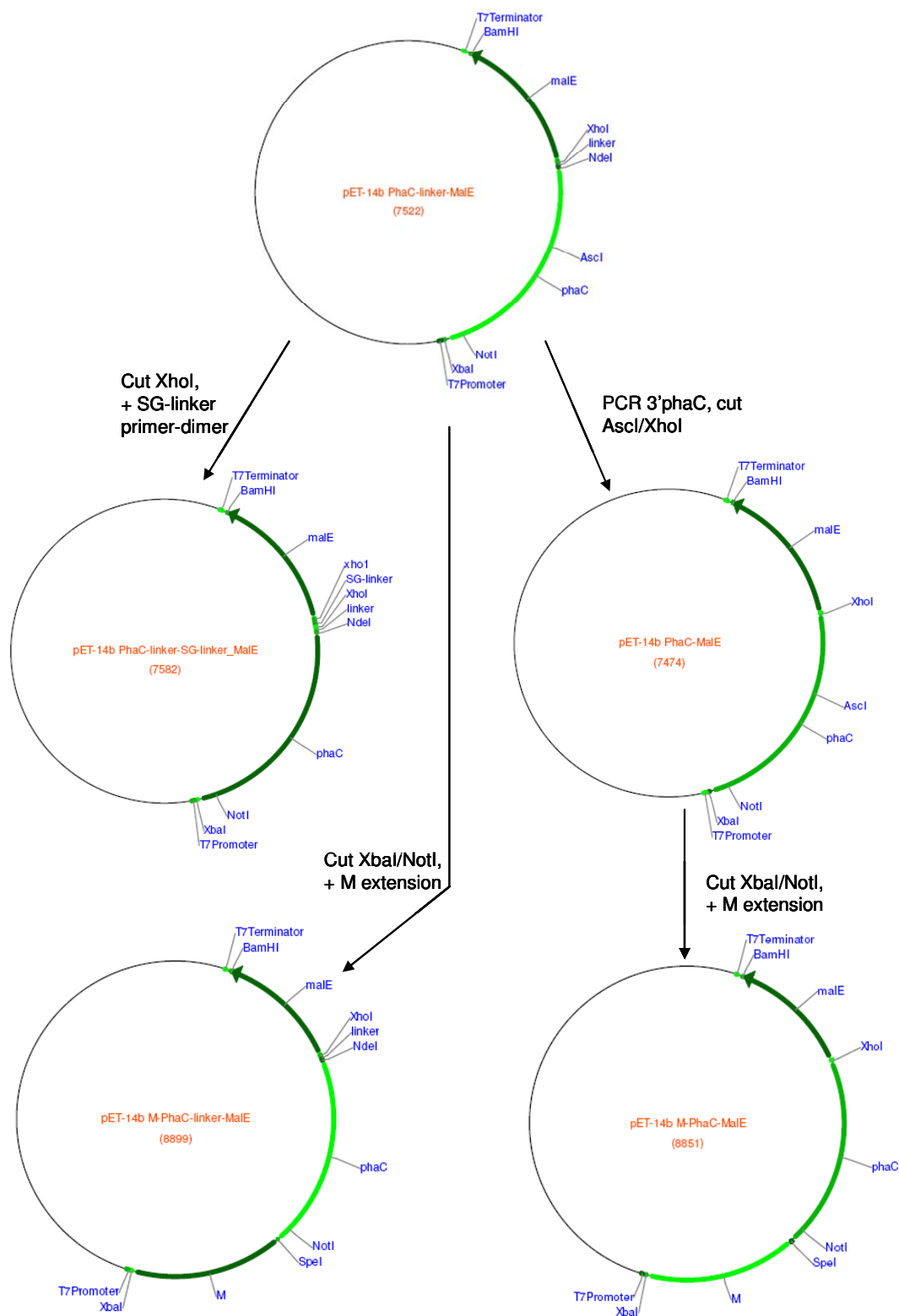
Figure 1. Schematic overview of the cloning strategy leading to plasmids encoding MalE fusion proteins. The plasmid size is indicated in base pairs in brackets. The detailed description is given in Chapter II.
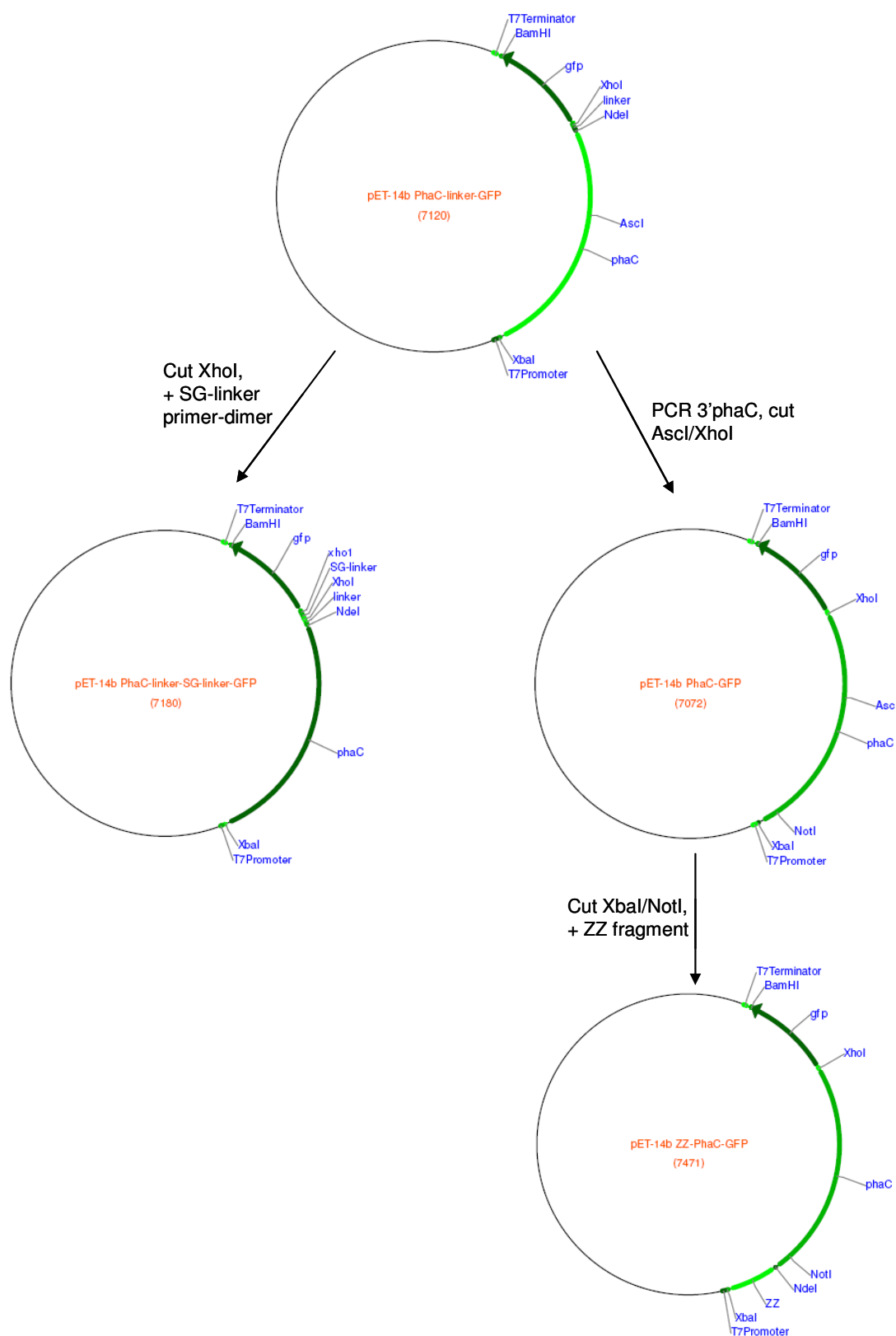
Figure 2. Schematic overview of the cloning strategy leading to plasmids encoding GFP fusion proteins. The plasmid size is indicated in base pairs in brackets. The detailed description is given in Chapter II.
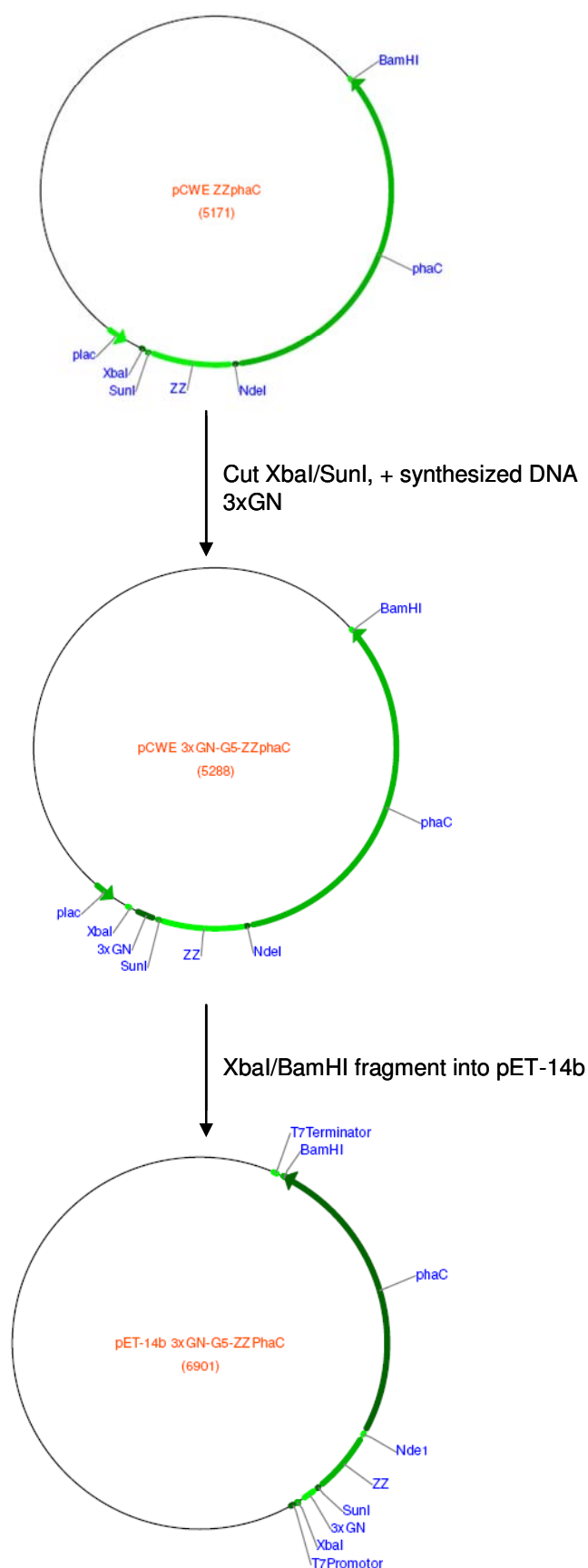
Figure 3. Schematic overview of the cloning strategy leading to the plasmid pET-14b 3xGN-G5-ZZPhaC. Similarly, the plasmid encoding the silica-binding synthase fusion was obtained. The plasmid size is indicated in base pairs in brackets. The detailed description is given in Chapter III.
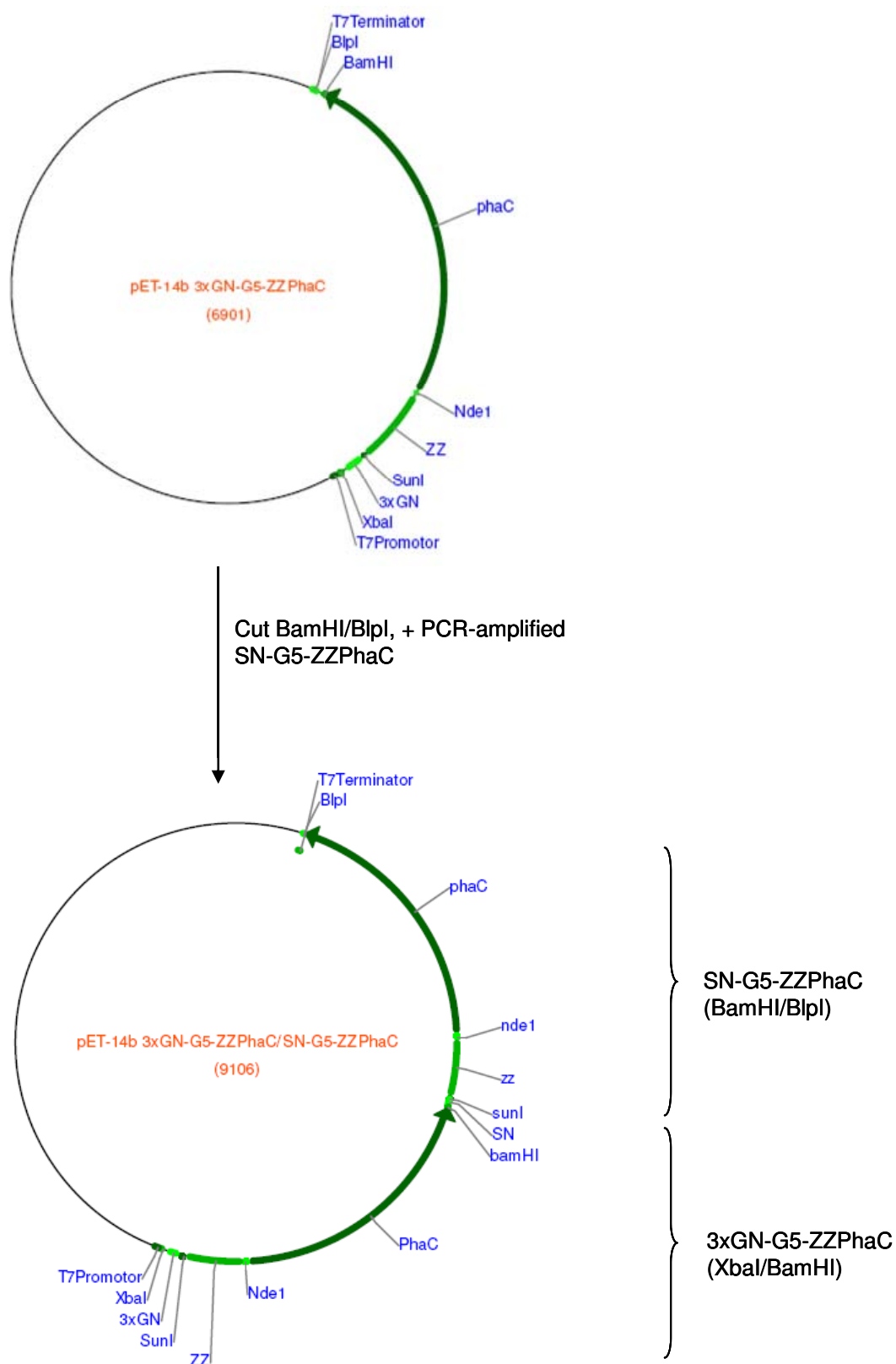
Figure 4. Schematic of the cloning strategy leading to the plasmid pET-14b 3xGN-G5-ZZPhaC/SN-G5-ZZPhaC, encoding two functionalised synthase proteins. The arrows indicate the individual fusion proteins. The plasmid size is indicated in base pairs in brackets. The detailed description is given in Chapter III.