Reviews

Bacterial Polyhydroxyalkanoate Granules: Biogenesis, Structure, and Potential Use as Nano-/Micro-Beads in Biotechnological and Biomedical Applications

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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 polymerization 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 that 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.

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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 artifact.

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.

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

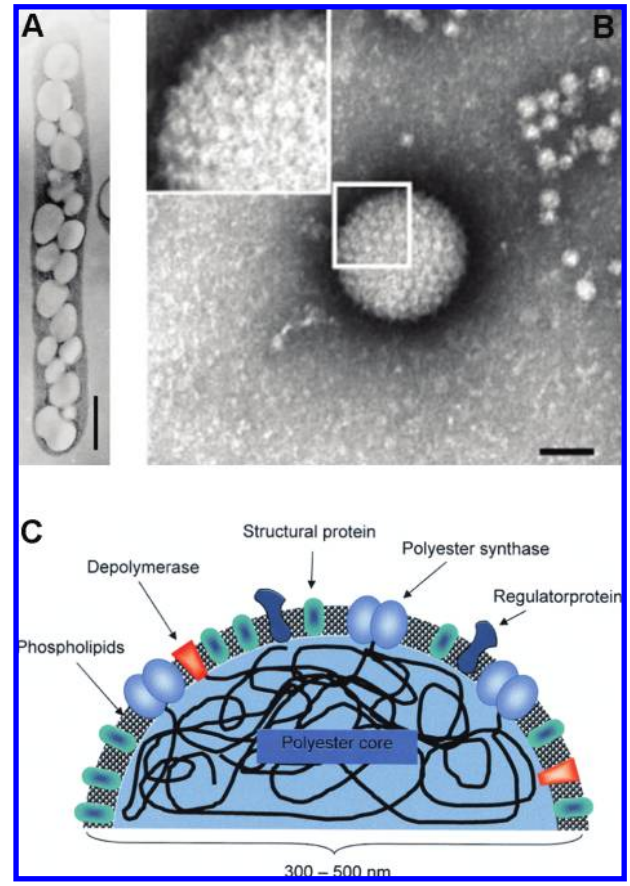

Figure 1. Different representations of PHA granules. (A) TEM image of *C. necator* cell filled with PHA granules. Bar, 500 nm (*Journal of Bacteriology*, **2005**, *187*, 3814−3824, doi:10.1128/JB.187.11.3814−3824.2005,[54] reproduced with permission from American Society for Microbiology). (B) TEM image 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. *Biochemical Journal*, **2003**, *376*, 15−33, DOI 10.1042/BJ20031254,[58] the Biochemical Society).

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 analyzing 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 nonrandom 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 stereoselective 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 40 kDa PhaC subunit and the other a 20 kDa PhaR subunit.[65] The PHA molecules synthesized 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 nonliving 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 nonpermissive 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 biobased 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]

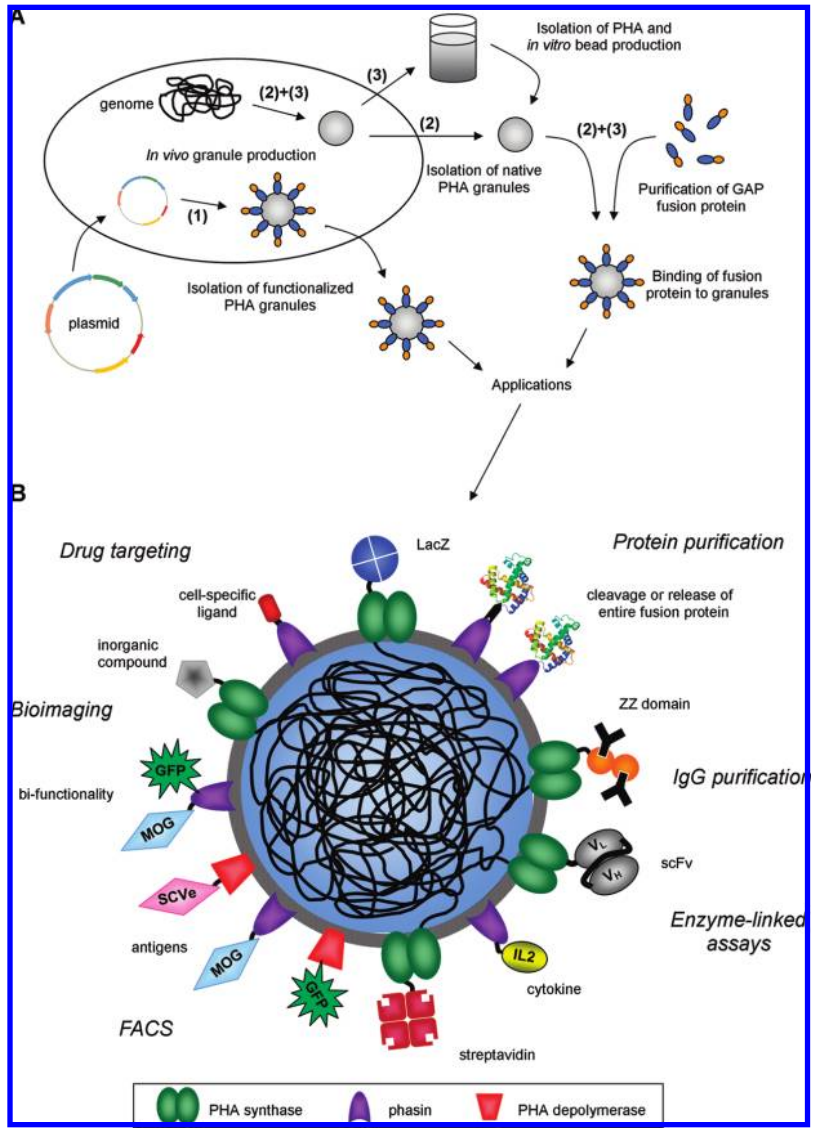

Figure 2. Potential applications for PHA granules. (A) Different approaches for the generation of functionalized PHA beads. (1) A plasmid encoded fusion of the target protein and a GAP is recombinantly produced in a PHA synthesizing host strain. (PHA synthesis can be natural or recombinant.) The fusion 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 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 coproduced 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 liter 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, and 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 toward 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.

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*. 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 functionalized 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 2 μm 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.

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