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Principles of Glycerol-Based Polyhydroxyalkanoate Production

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Abstract

The article addresses the contemporary quest for inexpensive carbon feedstocks to be used for cost efficient biomediated polymer production; such polymers can potentially be applied in the food technology sector, mainly for packaging purposes. In particular, the work shines a light on crude glycerol, a surplus stream of the globally tremendously emerging biodiesel industry. Crude glycerol can be upgraded to a convenient substrate for microbial polyhydroxyalkanoate production without interfering with food- or feed production. The article covers the challenges of using crude glycerol as a feedstock in biotechnology, and gives an insight into the metabolic background of glycerol-based polyhydroxyalkanoate production. Particularities of glycerol-based polyhydroxyalkanoate biosynthesis, such as the characteristic formation of low-molecular mass polyesters, and the resulting impact on polymer processing, are also discussed in this review.

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1. Introduction

Currently, the biopolymer market is highly dynamic and displays a remarkable increase regarding the range and volume of products; already in 2010, the market value of biopolymers reached a magnitude of 10^{10} USD with a clear upward trend. For example, from 2008 to 2015, the global annual production of so called "green plastics" is estimated to increase from 180 to 1710 kt [1].

In this framework, polyhydroxyalkanoate biopolyesters (PHAs) attract growing attention as biobased, biocompatible, and biodegradable "green plastics". This is due to their auspicious material properties, and their perfect embedding into nature´s closed carbon cycle; neither their production nor their utilization or disposal negatively impact the global ecosystem [2,3]. Biologically, these polyoxoesters of hydroxyalkanoic acids (HAs) serve as typical prokaryotic reserve compounds for carbon and energy. Factors usually boosting PHA accumulation are, analogous to the intracellular enrichment of other microbial storage compounds such as glycogen or polyphosphate, high intracellular energy charge, manifested by high intracellular concentration of the characteristic metabolites acetyl-CoA, ATP, or NAD(P)H, as reviewed by Koller et al. [4]. Such physiological situations result from ample supply of

the carbon source in parallel with sub-optimal concentrations of macro-components such as nitrogen, phosphate, sulfur, or oxygen, or micro-nutrients including, among others, different types of metals. The PHA molecules can be synthetized from various carbon-rich renewable resources by the biosynthetic action of selected prokaryotic microrganisms. The group of PHA-accumulating microrganisms comprises both Gram-negative and Gram-positive eubacteria, and an increasing number of archaea isolated from environmentally extreme habitats [5].

Depending on the applied carbon substrate and the bacterial or archaeal microorganisms, the material properties of PHA, recovered from microbial cells for further processing towards commercial items, can resemble those ranging from crystalline thermoplasts to flexible elastomers, latexes, and even smart, functional polymers. After their life span, items made of PHA undergo complete biodegradation to CO_2 and water as the sole products of microbial mineralization [6].

The precise material properties of PHA depend very much on their composition at the level of monomeric building blocks. In this context, highly crystalline, thermoplastic short chain length PHAs (*scl*-PHAs), constituting polyesters of HAs with 3 to 5 carbon atoms, are discriminated from rather elastomeric medium chain length PHAs (*mcl*-PHAs) which consist of HAs with 6 to 14 carbon atoms. Hence, based on their customizable properties, PHAs could be applied as a suitable alternative for their petrol-based counterparts in many segments of the plastic market [6,7].

2. Raw materials for PHA production

The implementation of White Biotechnology for production of frequently used materials such as (bio)plastics can be regarded as really encouraging for sustainable industrial development, although for a range of products, especially bulk-products, bio-technological production strategies still have not yet passed the threshold for economic feasibility. Especially the economics of PHA production is to a high extent determined by costs for the supply of raw materials; up to 50% of the entire production costs are allotted to the carbon substrates [8,9]. This is due to the fact that PHA accumulation takes place under aerobic conditions, resulting in high losses of carbon substrate by intracellular respiration. Hence, mainly due to a considerable loss of carbon by CO_2 formation through intracellular respiration and, to a minor extent, excretion of water soluble metabolites like organic acids, ionic polysaccharides, etc., only less than half of the carbon source is directed towards cell growth and PHA accumulation [9]. Typically, PHA production starts from expensive substrates of nutritional importance, such as refined sugars, starch, or valuable plant oils. Analogous to the contemporary "fuel vs. food" or "tank vs. plate" controversy, this provokes massive ethical conflicts since a high number of people are starving on our planet. Application of nutritionally important resources as raw materials for generation of biopolymers or biofuels increases food prices, thus acting as a driving force for inflation; this makes widely-available food nearly unattainable for numerous people [4,5]. As an alternative, upgrading of a broad range of carbon-rich industrial waste and surplus materials to feedstocks for biotechnology is suggested. Such materials, exemplarily listed in Table 1, are mainly produced in agriculture and the subsequent food technology sector. In the PHA case, the utilization of waste materials as feedstocks constitutes an auspicious strategy for cost-efficient and sustainable biopolymer production and, as a further benefit, supports agro- and food industry to overcome existing waste disposal problems, and even to generate financial profit from in-house waste [5,10,11]. As raw materials accruing at increasing quantities, crude glycerol phase (CGP) from biodiesel production or glycerol pitch from petro-chemistry has been recognized as a promising feedstock for PHA production and therefore constitutes the focus of this review. These surplus streams are accepted by a growing number of PHA-producing microbial strains as carbon- and energy source.

3. Background and particularities of glycerol-based PHA production

3.1. Increasing global quantities of biodiesel

In the last years, a tremendous increase in the production of biodiesel has caused a sharp decrease in the cost of glycerol, the main by-product of biodiesel synthesis [10,23]. This is caused by a new legislative situation in Europe and elsewhere that made addition of biofuels to common fuels compulsory. The increasing production of biodiesel in Europe generates enormous amounts of its major side stream, namely glycerol. In 2008, roughly 7-8 Mt of biodiesel was estimated to be produced in the EU, out of which 1-2 Mt was attributed to glycerol [23]. These amounts are by far higher than the industrial requirements for glycerol normally processed for a production of cosmetics, pharmaceuticals, food and chemicals. Hence, numerous alternative applications were investigated in order to upgrade the waste stream CGP to a feedstock for marketable (bio-) products. Besides PHA production, "green energy carriers" such as biogas, biohydrogen, and bioethanol are accessible from the fermentative conversion of glycerol by microbial consortia or by monoseptic microbial cultures. Other valuable compounds can be obtained by biotechnological conversion of glycerol including diverse organic chemicals like citric acid, docosa-hexaenoic acid, propionic acid, 3-hydroxy-propionic acid, succinic acid, dihydroxy-acetone, pigments, and substances for the chemical production of polymers like lactic acid, 2,3-butanediol or 1,3-propanediol [24,25].

Table 1. Inexpensive raw materials for PHA production

Surplus stream	Industrial branch generating the surplus stream	Selected references
Whey	Dairy industry, Cheese industry	[9,12]
Non-edible starch	Agriculture	[13,14]
Cellulosics and Lignocellulosics	Agriculture, Food industry, Paper industry	[15]
Methanol	Biodiesel industry	[16,17]
Methane	Biogas industry	[17]
CO_2	Industry in general, Biogas conditioning	[17]
Waste lipids	Animal processing industry, Biodiesel industry	[12,18, 19, 20]
Crude glycerol	Biodiesel industry	[9, 21,22]

3.2. Principles of biodiesel production and closing material cycles

Starting from lipids of different origins (herbal, animal, or microbial, see Figure 1), CGP accrues as a by-product of biodiesel production. Classically, biodiesel is generated in simple processes via alkaline-catalyzed transesterification (mainly KOH). The obtained CGP generally contains up to 60-70 $\%ww^{-1}$ of glycerol along with a variety of different impurities, mainly water, methanol, residual hydroxide, inorganic salts, fatty acids, and fatty acid mono- and diesters of glycerol [26]. It is a well-known fact that methanol represents a severe cell toxin for most microbial strains which inhibits microbial growth already at rather low concentrations; therefore, the selection of an appropriate production strain that tolerates a certain concentration of this alcohol is desired for a direct application of CGP without prior purification. As reported by Braunegg et al., this is the case for e.g., *Methylomonas extorquens*, an organism that, if cultivated in the presence of CGP, displays diauxic growth behavior by first converting methanol before glycerol is utilized as a carbon substrate [16]. Later, Moita et al. reported the parallel conversion of methanol and glycerol from CGP by mixed microbial cultures in a sequencing batch reactor [27]. After the carbon source in CGP is converted, PHA has to be recovered as an intracellular storage product by extraction or by disruption of the microbial biomass. Using established extraction techniques, microbial lipids can be used for subsequent transesterification cycles to produce biodiesel and CGP, whereas remaining biomass can be subjected towards hydrolysis, yielding a rich source of nitrogen, carbon and phosphate that can be used for microbial cultivation in another fermentation process [28]. Alternatively, residual biomass can be used as a "green fertilizer" in agriculture; the same application is possible for the phosphate-rich stream that remains after neutralizing the transesterification batch during biodiesel production. The main product of the transesterification, namely biodiesel, constitutes a well-known green energy carrier to be used in combustion engines. Alternatively, fine chemicals (e.g., surfactants) can be produced from biodiesel after chemical modification. Finally, low quality biodiesel fractions which do not meet quality requirements of biofuels, mainly obtained from animal-based lipids with a high content of saturated fatty acids, can directly be converted towards PHA by well-known lipase-expressing microrganisms like *Cupriavidus necator* [12,18,21] or *Pseudomonas* sp. [19,20]. The general scheme showing individual steps of the closed loop materials cycles, is presented in Figure 1.

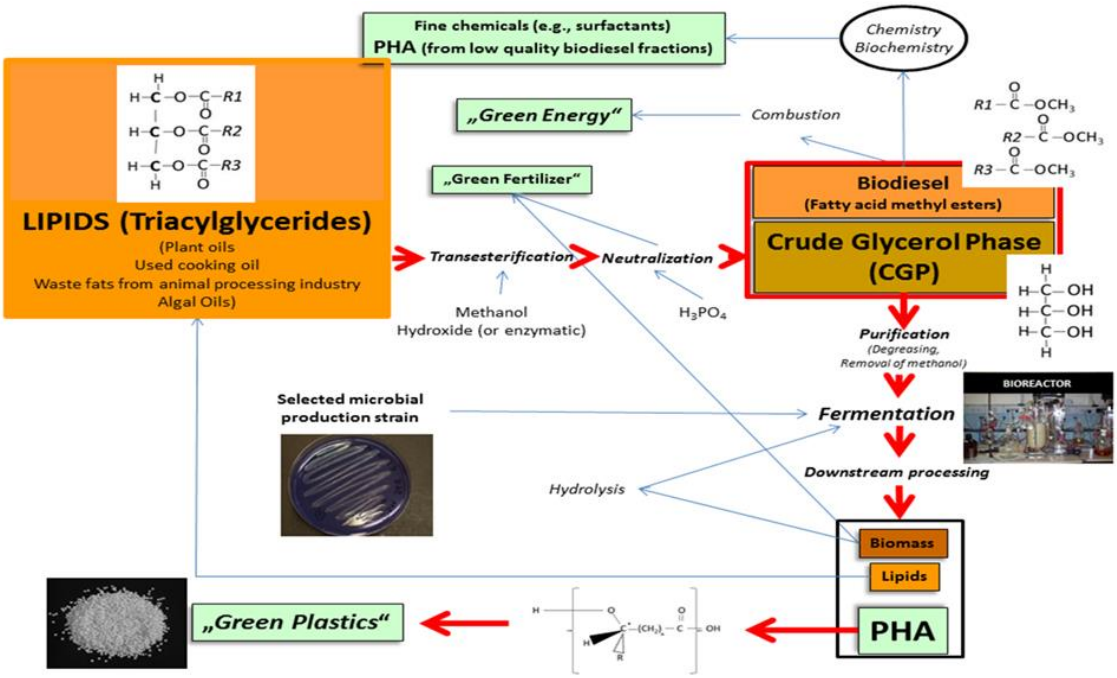

Figure 1. Scheme of PHA production from CGP from the biodiesel production. Red arrows indicate the main path from the raw materials (lipids) to the biopolymer as a final product.

3.3. Metabolic aspects

Due to the fact that the carbon atoms in glycerol are chemically in a higher reduced state of oxidation than in the case of glucose or lactose, cells using glycerol are generally in a more reduced physiological state; this favors the intracellular PHA synthesis. In general, glycerol can be regarded as one of the most favorable substrates for generation of the PHB-precursor acetyl-CoA. On bioreactor-scale, Rodriguez-Contreras et al. used glycerol and glucose in a co-feeding strategy to produce PHB homopolyester by two different production strains, namely *C. necator* DSM 545, and *Burkholderia sacchari* DSM 17165. Especially the impact of the switch from glucose to glycerol as a carbon source on growth kinetics, polymer production, and molecular mass of the produced polyesters was studied. Although considerable cell masses were obtained for both micro-organisms, *C. necator* turned out to be a far more promising candidate for glycerol-based PHB production in comparison to *B. Sacchari* [22].

The next paragraphs give a short insight into the importance of choice of the carbon source for overall PHA productivity, mainly focusing on the particularities of using glycerol as a carbon source in comparison to other substrates e.g. sugars.

In a study carried out by Santhanam and Sasidharan, several carbon sources (sucrose, fructose and lactose and *n*-octane) were tested for a PHA production in *Alcaligenes eutrophus* (today: *C. necator*), *Alcaligenes latus* (today: *Azohydromonas lata*), and *Pseudomonas oleovorans* under nitrogen-limitation [29]. The authors concluded glucose to be the most appropriate carbon substrate for maximum PHA accumulation in *A. eutrophus* reaching 4.14 gl^{-1} PHA, whereas slightly lower PHA yields were recorded for A. latus supplied with sucrose. Due to different metabolic capabilities, *P. oleovorans* produced low PHA concentration with sugars, but was the only strain able to utilize n-octane (2.06 gl^{-1} PHA production). In another Pseudomonas strain (*P. putida* CA-3), Ward and O'Connor found phenylvaleric acid to be the most suitable carbon source out of 6 tested phenylalkanoic acids, significantly increasing yield and PHA content, namely 0.59 g PHA per g cell dry mass (CDM) [30], while in another experimental setup with *P. putida* SU-8, only slight differences in PHA mass fraction in CDM were found comparing glucose and lactose grown cells (0.36 gg^{-1} and 0.35 g g^{-1}, respectively) [31]. Feeding of cells with various carbon sources can therefore significantly enhance PHA production and accumulation, but is also decisive for the PHA composition [32] and expression levels of genes involved in different metabolic pathways such as Embden-Meyerhof-Parnas pathway (EMP), Entner-Doudoroff pathway (ED), TCA, fatty acid *de novo* synthesis, or gluconeogenesis [33,34]. Figure 2 provides a simplified schematic of the major metabolic pathways from glycerol to PHA. The import of glucose as the most common carbon substrate for PHA production is additionally indicated.

3.4. Impact of glycerol on the molecular mass of PHA

As a particularity, PHAs obtained from glycerol were reported to have significantly lower molecular masses than polymers synthesized from other substrates, typically less than 1,000,000 Da. Hence, if glycerol is applied as a raw material for PHA biosynthesis, one has to consider the suitability of these extracted polymers for intended purposes. For certain special fields of application, low molecular mass PHA could be desired, i.e. for utilization as softeners in (bio) polymeric blends, for food packaging or for medical devices [35]. In *M. extorquens* and *R. eutropha* (*C. Necator*), PHB obtained from glycerol, ethanol, or methanol had a lower average molecular mass (Mw) than that obtained from other substrates (such as succinate, glucose, and fructose); the Mw of the polymers is decreased with increasing glycerol concentrations [36]. This generation of polymers with low molecular masses was further investigated in details. As was revealed, glycerol or similar compounds present in the medium can terminate the propagation of polymer chains of growing PHA molecules. Mechanistically, this happens by covalent linking of hydroxyl groups at the carboxyl terminus of the polyester. This mechanism is called "endcapping" or "chain termination" effect as described in the work of Madden et al. [37]. Pioneering studies accomplished by Ashby et al. reported this effect for PHA produced by different Pseudomonas strains. In these studies, Mw of the accumulated polymers decreased with increasing glycerol concentrations both in the case of Mw of PHB produced by *P. oleovorans*, as well as in the case of *mcl*-PHAs accumulated by *P. corrugata* [38]. In the same year, similar findings were also reported for haloarchaeal PHA production by Koller et al., who for the first time described the dependence of molecular mass of archaeal PHA on the applied carbon source. The investigated production strain, *Haloferax mediterranei* DSM 1411, an extremely halophile representative of the Euryarchaeota division, produced a PHBHV copolyester with Mw of 253,000 Da if cultivated on CGP, but of 696,000 Da if grown on sugars stemming from hydrolyzed whey lactose. Independent of the applied carbon source, both polyesters displayed a homogenous molecular mass distribution as reflected by their low polydispersity indices (PDIs) which was reported to be significantly below 3 [9]. These findings were later confirmed by the generally low molecular masses and low PDIs of different glycerol-based archaeal PHA co- and terpolyesters as reported by Hermann-Kraus et al. [21]. Molecular mass analysis of the PHA produced from CGP by *Novosphingobium* sp. THA-AIK7 revealed a PHB homopolyester of considerably low molecular mass (Mw 23,800 Da) and a high PDI of 5.6, indicating a highly heterogeneous material regarding the molecular mass distribution [39].

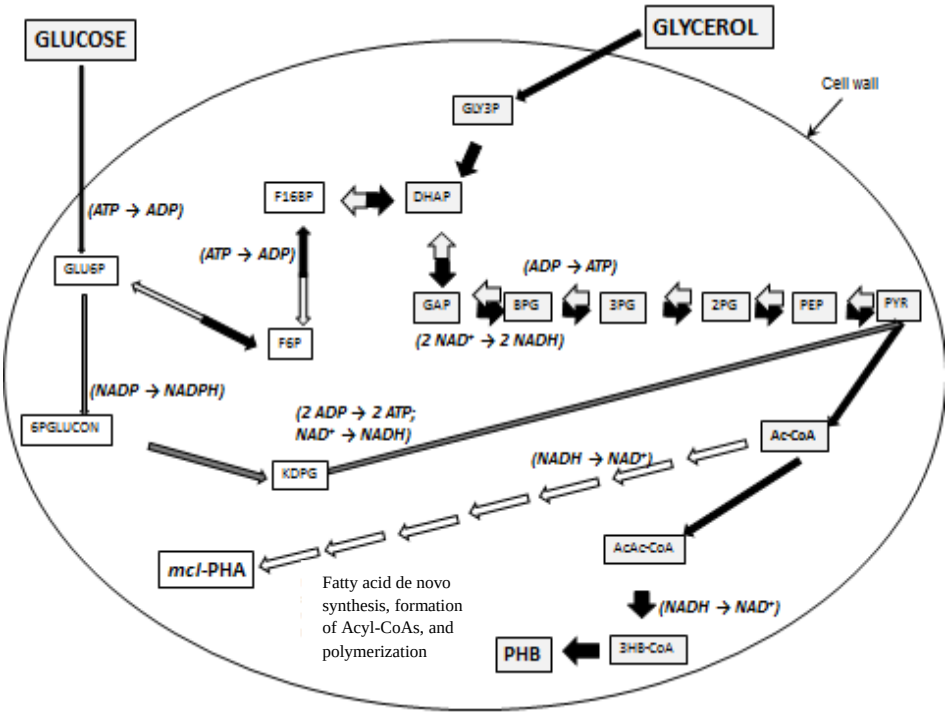

Figure 2. Simplified schematic of metabolic pathways from glycerol to PHA. Black arrows: Emden-Meyerhof-Parnas pathway (EMP, glycolysis); light grey arrows: gluconeogenesis; dark grey arrows: Enter-Doudoroff pathway (ED, KDPG-pathway); white arrows: *mcl*-PHA formation based on fatty acid *de novo* synthesis. GLY3P: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde phosphate; BPG: bisphosphoglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; KDPG: 2-keto-3-desoxy-6-phosphogluconate; 6PGLUCON: 6-phosphogluconate; GLU6P: glucose-6-phosphate; F6P: fructose-6-phosphate; F16BP: fructose-1,6-bisphosphate; Ac-CoA: acetyl-CoA; AAC: acetoacetate; AcAc-CoA: acetoacetyl-CoA; 3HB-CoA: 3-hydroxybutyryl-CoA.

Biopolymers obtained by cultivating *C. necator* and *B. sacchari* on glycerol revealed low molecular masses of about 300,000 Da (PDI 4.72) with *C. necator*, and of about 200,000 Da (PDI 2.50) with *B. sacchari*, respectively [22]. Using the strain *B. sacchari*, Zhu et al. reported a gradual decrease of molecular mass from 304,000 to 162,000 Da with increasing glycerol contents (3 to 9%) in feed streams containing both glucose and glycerol as carbon substrates [40]. Also in the work of Tanadchangsaeng et al., who cultivated *C. necator* ATCC 17699 on glycerol, NMR analysis revealed the "endcapping" of PHB by glycerol; again, the final molecular mass of the glycerol-based polyester was lower than the molecular mass of the glucose-based one, although similar thermal and mechanical characteristics of both materials were reported. These authors also observed the ongoing decrease of molecular mass during longer cultivation time [41]. Similar observations were confirmed in a study involving *mc*l-PHA production by *Pseudomonas* spp. *P.mediterranea* 9.1 (CFBP 5447) from pure glycerol or partially refined CGP where low molecular masses of the polymer ranging from 56,000 to 63,000 Da were reported [35]. Tsuge et al. also observed that, similar to the previous reports, higher glycerol concentration, even when added in a chemically bound form as a component of triglycerides, resulted in considerable reduction in molecular mass of PHA. With the highest concentration of glycerol tested (10 gl^{-1}), molecular mass of accumulated PHA decreased by 54%. Also these authors assumed that this effect was caused by a chain transfer reaction catalyzed by glycerol ("endcapping") which promoted termination of the phaC polymerization activity. Using recombinant *R. eutropha* harboring the PHA synthase gene from *A. caviae* (*phaCAc*), the study also showed that various alcohols, structurally related to glycerol (e.g., glycols), also promoted the chain transfer reaction. This reaction was found to be more significant with higher hydrophobicity, hence the length of the alcohol applied. For example, in the presence of 1 mM ethanol (low hydrophobicity), this effect did not occur, while in media supplied with 1mM 1-butanol or 1-hexanol, the molecular mass was drastically reduced by 57 % and 70 %, respectively. Because higher molecular mass provides PHA with higher mechanical strength and therefore better material properties, raw materials should be checked for alcohol contamination as a quality control of PHA molecular mass [42].

Also exceptions to these findings are found in literature: Mothes et al. reported that the molecular mass of PHB produced with *C. necator* or *Paracoccus*

denitrificans from crude glycerol varies between high values of 620,000 and 750,000 Da, respectively, which might already allow the processing by common techniques of the polymer industry, although these values are still somewhat lower than typical molecular masses of sugar-based PHB [43]. Similar conclusions were reported in a study with recombinant *R. eutropha* grown on glycerol [44] where no molecular mass reduction of PHB was confirmed as previously explained [42]. In addition, Cavalheiro et al. reported astonishingly high molecular masses of Mw between 550,000 and 1.370,000 Da for *C. necator* cultivated on CGP supplemented with precursors for co- and terpolyester synthesis [45]. Results of this study are coherent to prior experiments of this group, reporting Mw of glycerol-based PHB from *C. necator* from 790,000 to 960,000 Da [46].

4. Conclusion

Regarding the low molecular masses of most glycerol-based PHAs, it has to be decided what applications these materials are suitable for in terms of processing via e.g. injection molding or melt extrusion where a decrease in molecular mass can significantly impact their material properties. In regard to processing, data for these materials are still completely missing! Nevertheless, some production strains, especially *C. necator* ssp., accumulate PHA with molecular masses not too different from sugar-based polyesters. For other strains, the co-feeding with other carbon sources could considerably preserve the molecular masses. Alternatively, PHA of low molecular mass might act as a suitable filler material for biopolymeric composites and blends.

It has to be emphasized again that the increasing quantity of surplus glycerol from biodiesel manufacturing urgently requires new value-added ways of application. Regarding the available data from lab-scale, sustainable microbial biopolyester production appears to be among the top strategies of choice to combine new solutions for industrial disposal problems with value-addition.

5. Acknowledgments

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6. Conflict of interest

The authors declare that there exist no conflicts of any interest.

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