Rapid Assay of Plastic Biodegradation Using Simply Fabricated Polyethylene Nanoparticles

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Abstract

Synthetic polymers, such as polyethylene (PE), are produced daily in large quantities and due to their unique properties, like durability and low cost, consumed in various capacities around the world subsequently accumulating in terrestrial and marine environments. In recent years, the toxicological effects of these plastic particles on the environment, food chain, and public health as well as bioremediation approaches for the removal of these wastes has been the focus of several studies. Easy access to nano and micro sized PE particles in these approaches is crucial and urgent. In this research, we report a simple, fast, cost-effective method for the production of nano polyethylene particles (NPE) which are stable, capable of dispersion in water and without chemical modifications. PE particles were characterized by dynamic light scattering (DLS), X-ray diffraction (XRD), scanning electron microscopy (SEM) and Fourier Transform Infrared (FTIR) analysis. As a result, particles were made with a wide range of sizes 72-1005 nm (but mostly at size of 500 nm) with PDI of 0.88. The non-toxic nature of these nanoparticles for microorganisms were tested by MTT assay.

The relevance of this method for the production of NPE is especially prominent in toxicity assessment studies which evaluate the effects of these particles in living organisms. Moreover, this method can be used to generate particles for efficient selection of PE-degrading microorganisms from the environment and scaled up for industrial use to facilitate the degradation process of PE and conversion of wastes to high-value products.

Introduction

For decades now, chemical processes for the production of synthetic polymer has led to the formation of new materials with a combination of strength, flexibility, durability, low cost and lightness qualities which make them useful in various applications [1]. Polyethylene (PE) consists of long chains of carbon and hydrogen atoms with balanced charge, various degree of branching and lack of functional groups [2] and is mainly used in the packaging industry since it is disposable and recalcitrant to degradation. Excessive consumption of the petroleum-based polymer leads to accumulation the waste in the different environments such as oceans, soil and even the arctic circle [3–5].

Microorganisms have important and key roles in various ecosystems and are also involved in natural and synthetic polymer degradation [6, 7]. The process of microbial degradation studies usually involves the isolation of microorganisms from various sources using culture-dependent methods and analysis of their enzymes for plastic degradation. However, due to the durable structure PE polymer and also the history of studies in this field, unfortunately, the time period to assay plastic degradation potential is long (at least 1 month) [8] which could however be shortened physical and chemical treatments.

In general, there are two main strategies for polymeric nanoparticle production. First, the dispersion of polymers and second, monomer polymerization [9, 10]. In recent years, some efforts have been directed at constructing nano or micro-PE particles alone or in composite with other material, for various purposes.

However, there is a need for PE nanoparticles without additives and modifications in its properties in protocols leading to the screening of environmental microorganisms for PE degradation. Furthermore, a PE nanoparticle preparation method with fast, easy and cost-effective features introduced here demonstrate powerful potentials for use in toxicity studies.

Material & Methods

Construction of PE nanoparticles

One g of commercial (0190) low density polyethylene (LDPE) powder was dissolved in 10 ml of xylene (Merk) [11] at 100 °C for 30 min until no solid PE was visible. The solution was cooled rapidly at 0 °C, 100 ml icy deionized water added [12], and vigorously mixed together, to emulsifying which was immediately subjected to ultrasonication (S-4000-010, Misonix) or ultrasonic bath for 1h in an ice water bath. NPE were suspended in water following which PE residues were filtered with Whatman Grade 1 membrane (Whatman, USA). To remove xylene, the mixture was centrifuged at 20000 x g for 30 min and the supernatant decanted. In order to vaporize the remaining xylene molecules, the tubes containing the precipitated particles were placed in the oven at 80 °C for 30 min. Subsequently, deionized water was added and the PE particles were washed from the tube walls and autoclaved. Finally, a clear mixture of NPE was obtained which indicated dispersion of these particles in water. In fact, NPE were made by emulsification-solvent evaporation method [13].

NPE particle characterization

The particle size distribution was identified by dynamic light scattering on Sympatec GmbH, NANOPHOX [14]. Scanning electron microscopy (SEM) was used to evaluate the size, shape and aggregation of particles on Quanta200 ESEM, FEI [15]. Freeze-dried NPE particles and PE powder tablets were prepared using potassium bromide (KBr) powder were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) to detect changes in the functional groups on their surface [16]. X-ray diffraction (XRD) technique was utilized for phase identification of crystalline and amorphous NPE on Ultima (Rigaku, Japan) [17, 18].

Effect of PE particles toxicity on bacterial growth

The nanoparticles were used as the sole carbon source in the formulation of a Bushnell Hass (BH) mineral medium for cultivation of bacteria [3]. Bacterial strains were selected for analysis and inoculation of the relevant culture media. *Rhodococcus ruber* IR-SGS-T6 [19], previously shown to degrade PE and isolated from a landfill in Iran, was used as positive control in present research. The other bacterium was EPI300TM-T1^R *E. coli* as negative control, recombinant EPI300TM-T1^R *E. coli* carrying a construct containing gene sequences encoding hydrolyzing enzymes from a fosmid expression library. The recombinant EPI300TM-T1^R *E. coli* had been selected on the basis of the ability to degrade PE as the sole carbon source in the medium (unpublished data). Bacteria were cultured in Luria Bertani (LB) broth and allowed to grow to meet a concentration of 3×10^8 CfU/ml (equivalent to OD_{λ600} 0.25). Two milliliter of

each bacterial suspension were centrifuged in 3500 x *g* for 10 min to obtain cell pellets, which were then resuspended in equal volume of fresh mineral medium (BH). One percent of the volume from each bacterial suspension was inoculated to mineral medium supplemented with NPE particles as sole carbon source and incubated in a shaker incubator at 37 °C and 150 rpm for a 5-day period. The optical density (OD) of bacterial suspension at 600 nm was measured immediately after inoculation and at the end of incubation period using spectrophotometer. Scanning electron microscopy was used to envision the growth of microorganisms in the culture medium.

MTT colorimetric assay was performed to assess NPE toxicity on bacterial metabolic activity [20, 21]. Aliquots of 150 µl of each bacterial suspension and 10 µl of MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide] (Sigma) solution (5 mg/ml) were added to wells in microplate, mixed together and incubated at 37 °C for 1 h. The emergence of purple color was considered as the measure of bacterial cell viability and metabolic activity, consequently assumed as the non-toxicity of PE particles.

Results

Characterization of NPE Particles

As a result of PE polymer dissolution in solvent and ultrasonication, a white turbid mixture was obtained which became a clear and colorless solution following purification and removal of the organic solvent. DLS analysis, essential in evaluating the size and shape distribution of the NP, was carried out subsequent to which the hydrodynamic diameter was determined to be 422.81 – 1005.63 nm. Polydispersity index (0.88) indicated a very broad particle size distribution [22](Fig. 2a).

A more precise estimation of size and shape of particles were ascertained by SEM images. Since PE particles are inherently highly hydrophobic and tend to aggregate, they show a high degree of variation in size and shape (Fig. 2b).

XRD pattern of NPE against original PE polymer shows during the production of these particles, the crystalline phase is reduced and most of the resulting particles are amorphous (Fig. 3).

Surface properties of PE powder (as control) and constructed particles were analyzed by FTIR spectrometry. Comparison of the resulting spectra revealed that almost no changes were made in their surface during the process of NPE particles production and these particles had same surface structure of PE powder. As shown in Fig 4, the peak marked at 3446-3448 cm⁻¹ represents wet KBr O-H bond.

The optical density (OD), as an indication of bacterial growth, was measured at 600 nm. At time zero, OD of both native and recombinant bacteria was recorded to be 0.01 and following five days of incubation *Rhodococcus ruber* IR-SGS-T6 culture reached OD of 0.3 and recombinant *E. coli* culture reached 0.13, clearly demonstrating bacterial growth as a result of NPE consumption by the microorganisms in the culture media as sole carbon source. Scanning electron microscopy (Fig.5) of both bacterial cultures showed growth and presence of inoculated bacteria with NPE particles

MTT assay was carried out 5 days after incubation to assess the toxicity of the particles. In this assay, EPI300TM-T1^R *E. coli* strain (host bacteria in recombination) served as negative control, *Rhodococcus ruber* IR-SGS-T6 as positive control and recombinant EPI300TM-T1^R *E. coli* were used. Both *Rhodococcus ruber* IR-SGS-T6 and recombinant EPI300TM-T1^R *E. coli* were viable as indicated by the presence of dehydrogenase and reductase activities in the bacterial which reduced yellow soluble tetrazole to purple formazan crystals (Fig. 6).

Discussion

PE is a very recalcitrant polymer, resistant to degradation process due to the presence of the linear backbone of carbon atoms, degree of crystallization and high molecule weight, hydrophobicity, and insolubility in water and remains in the environment for several years even after exposure to moist soil or water [23]. Several studies have been carried out on microorganisms and enzymes capable of PE polymer degradation and their mechanisms. However, the resistant structure of PE eludes short term analysis [24]. NPE particles are beneficial and convenient in tests for rapid screening of microorganism involved in plastic degradation and relevant enzyme assays. Native degrading microorganisms can easily colonize, form biofilms and hence degrade resistant polymer films or their fragments, but engineered bacteria carrying genes coding for relevant hydrolytic enzymes do not have the potential for attachment and biofilm formation and hence the use of NPE particles is recommended for the study of heterologous enzymes. For example, Wei and coworkers used nanoPET particles for the enzymatic hydrolysis assay of polyethylene terephthalate (PET) [25]. Danso and colleagues used nanoPET to test engineered bacteria with synthetic genes obtained from metagenomics databases for PET degradation function detection [26].

On the other hand, plastic particles with a size of <5 mm released in terrestrial and marine environments eventually enter the food chain and cause ecotoxicological problems in living organisms [27, 28] and therefore, the exploitation of these nanoparticles could be valuable in the design of various evaluation experiments [29].

Synthesis of PE magnetic nanoparticle using decalin solvent and tetraglyme as a nonsolvent emulsion and high temperature for generating crystallization by ultrasonication as well as CTAB surfactant for drug delivery has been previously reported [30]. Kolb and colleagues used a solution of nickel complex as a catalyst in small amount of solvent and surfactant for diaper ethylene in the aqueous medium under temperature and pressure to form lattices with average sizes 100 nm to 1 μ m [31]. A method for PE microplastics was developed by Balakrishnan and coworkers dissolving the polymer in toluene after which it was emulsified in water and surfactant, and particles were dispersed by ultrasonication. Nanoparticles were then used to evaluate the toxicological effect of PE microplastics on marine biota [29]. In our study, surfactants as additives were excluded from experiments since the effects of PE on bacterial growth was being assessed. On the other hand, xylene was used as a solvent as it is volatile and is rapidly removed from particles. This method is simple and reliable and does not necessitate any specific instrumentation.

Polymeric nanoparticles are defined in the size range of 1-1000 nm [10, 32] and as the results from DLS and SEM analysis indicate, NPE particles show a size range between 70 and 1005 nm, and a PDI of 0.88 which signifies a wide range of size distribution and polydispersity. Since these particles are hydrophobic, they tend to bind together and therefore, it is deemed possible smaller particles join to form larger particles seen as two peaks in the DLS result (Fig. 2a). These peaks represent two population of particles with two different sizes of 507.65 nm and 810.35 nm which are predominantly present in the sample.

Reducing the size of the polymer increase available surfaces for biodegradation [1]. Based on the results obtained from XRD and SEM analysis, NPE particles have amorphous forms making them more vulnerable and accessible to degradation compared to the crystalline structure [33, 34].

As results from this analysis indicate, the technique presented in this paper for the production of nano-PE particles dispersed in water is simple, easy, and cost-effective and could be exploited for the implementation of experiments investigating the toxic effects of nano-PE on living organisms. Furthermore, these particles could be valuable in the detection and efficient use of microbial cell factories to convert PE plastic wastes into high-value biopolymers such as biopolymers (PHA), providing an alternative to conventional recycling and hence, promoting the wellbeing of the environment [35, 36].

Conclusion

We report an effective and simple method for NPE particles production with no apparent chemical changes on the surface. The resulting products are nano in size, stable and dispersible in water. These particles could be useful for studies involving PE degradation by microorganisms and possibly, assessment of nano plastic toxicology for *in vivo* and *in vitro* studies.

Declarations

Conflict of interest:

The authors declare that they have no conflict of interest

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Figures

Figure 1

Schematic description of the method used to preparation of NPE particles

a

Polyethylene

$x_{10} = 422.81 \text{ nm}$	$x_{50} = 507.60 \text{ nm}$	$x_{90} = 844.48 \text{ nm}$	$SMD = 535.98 \text{ nm}$	$VMD = 599.12 \text{ nm}$
$x_{16} = 440.07 \text{ nm}$	$x_{84} = 810.35 \text{ nm}$	$x_{99} = 1005.63 \text{ nm}$	$S_V = 11.19 \text{ m}^2/\text{cm}^3$	$sd = 185.93 \text{ nm}$

Width = 530.69 nm PDI = 530.69 nm / 599.12 nm

b

Figure 2

PE particle size distribution, **(a)**. DLS (dynamic light scattering) result of NPE particles, **(b)**. SEM images of NPE particles and different apparent aggregates of hydrophobic particles.

Figure 3

X-ray diffraction (XRD) **(a)**. PE powder with crystalline two sharp band in the 20° - 23.5° 2θ range, **(b)**. NPE particles in amorphous form with broad band in the 5° - 80° 2θ range.

Figure 4

FTIR spectrum of PE powder, NPE particles and KBr. The marked peak in the graph is related to H-O bond in wet KBr

a **b**

Figure 5

Scanning electron microscopy, **(a)**. Native bacteria (*Rhodococcus ruber*), **(b)**. Recombinant *E. coli* in PE-MS media culture.

Figure 6

MTT assay of EPI300TM-T1^R *E. coli* strain as negative control, *Rhodococcus ruber* IR-SGS-T6, Recombinant *E. coli* as positive clone.