

POLYMER MICROPARTICLE-BASED SCREENING PLATFORM FOR PLASTIC DEGRADING ENZYMES

Field of Invention

The present invention generally relates to screening platforms, and more particularly relates to a polymer microparticle-based screening platform for plastic degrading enzymes.

Background

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Over the years various microorganisms capable of degrading certain forms of plastic were identified and the enzymes responsible for the degradation were characterized. These plastics typically include polyethylene (PE), polyethylene terephthalate (PET), polyurethane (PU), polystyrene (PS), among others. Incidentally, a discovery made by Yoshida *et al.* on a novel bacterium – *Ideonella sakaiensis* which was isolated from samples obtained around a PET bottle recycling factory in Japan in 2016 has garnered widespread attention. Unlike the previously described enzymes, this one was active at ambient temperatures. This bacterium grows on PET as carbon source and secretes enzyme – PETase and MHETase. PETase enzyme can degrade PET into 2-Hydroxyethyl terephthalic acid (MHET) and terephthalic acid (TPA) with some traces of Bis(2-Hydroxyethyl) terephthalate (BHET) and further MHETase can cleave the MHET into the respective monomer (Yoshida, S., *et al.*, *Science* **2016**, 351(6278), 1196-1199; and Austin, H. P., *et al.*, *PNAS* **2018**, 115(19), E4350-E4357). Ever since, various labs across the world have been working towards engineering this enzyme as the activity of the identified enzyme is rather low. Some improved variants of this enzyme have since been published using rational protein engineering and mutating specific amino acids. The process of identifying an improved mutant is mainly limited by the availability of tools that can screen the enzymes from a library of generated enzymes. The current methods of identifying and screening various plastic degrading enzymes utilize high-performance liquid chromatography (HPLC) analysis which is cumbersome and simply impossible to apply to the vast array of mutants that are generated in mutant libraries.

The earliest methods used for measuring activity of enzymes include gravimetric method where change in mass of substrate upon treatment is measured and qualitative analysis using

SEM imaging. In these quantitative methods, the substrate has to be retrieved after reaction for analysis. The current method of analyzing the activity is by determining the degradation products released using HPLC analysis. HPLC method of analysis provides precise concentration of degradation products but the method requires clean sample for analysis thus a need for prior sample preparation step which makes this method the most labor intensive and discontinuous while requiring several minutes for the analysis of a single sample.

Some indirect methods have been developed for analysis such as turbidity measurement of PET nanoparticles suspension upon enzymatic reaction. And for a faster detection of enzyme activity, model substrate like p-nitrophenyl acetate is used which provides spectrophotometrically detectable product, p-Nitrophenol (pNP). The dissimilarities between actual PET substrate and model substrate makes for an unreliable comparison between the pNPA activity with actual degradation of PET. There is also a quick saturation in pNPA assay which renders it irrelevant to screen for enzymes with high activity as the difference in activity becomes undistinguishable.

Another indirect method of analyzing the PET degradation includes titration method. As the enzyme-substrate reaction leads to formation of acidic products (MHET and TPA), the amount of base to neutralize the acidic solution provides the estimation of product concentration and thus the enzyme activity, however the method fails to account for BHET as it is not acidic. Recent advancement attained in continuous and faster analysis of activity is by bulk absorbance method in which the degradation products are detected by obtaining a linear absorbance profile of the degradation products using spectrophotometer which cannot be measured using crude enzyme lysates.

Therefore, there exists an urgent need for the development of platforms that can significantly reduce the time and steps required for screening plastic degrading enzymes.

Summary of Invention

Aspects and embodiments of the invention are provided in the following numbered clauses.

1. A polymeric microparticle comprising:
a polymeric material that is susceptible to enzymatic degradation; and
a dye encapsulated within the polymeric material.

2. The polymeric microparticle according to Clause 1, wherein at least a portion of the dye is released from the microparticle in an environment containing an enzyme suitable for degrading the polymeric material.
3. The polymeric microparticle according to Clause 1 or Clause 2, a polypropylene (PP), a polybutylene adipate terephthalate (PBAT), a polyamide (PA), and more particularly, a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), a polyethylene (PE), a polyurethane (PU) and a polystyrene (PS), copolymers thereof and blends thereof.
4. The polymeric microparticle according to Clause 3, wherein the polymeric material is selected from the group consisting of a polyamide (PA), a polystyrene (PS), a polyester-polyether-polyurethane-copolymer (PU/PCL copolymer); and a polyethylene terephthalate and polybutylene terephthalate blend (PET-PBT blend), and, more particularly, a polystyrene (PS), a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), and a polyethylene (PE).
5. The polymeric microparticle according to Clause 4, wherein the dye is a fluorescent dye, optionally wherein the dye is selected from one or more of the group consisting of methylene blue, toluidine blue O, acid fuchsin, alcian blue, sulforhodamine, and more particularly rhodamine B, and acridine orange.
6. The polymeric microparticle according to any one of the preceding clauses, wherein the dye is selected from one more of the group consisting of methylene blue, toluidine blue O, acid fuchsin, and more particularly, acridine orange.
7. The polymeric microparticle according to Clause 6, wherein the dye is acridine orange.
8. The polymeric microparticle according to any one of the preceding clauses, wherein the dye is present in an amount of from 0.5 to 3 wt%, such as from 0.6 to 2 wt%, such as from 0.61 to 1.93 wt%.
9. The polymeric microparticle according to any one of the preceding clauses, wherein the microparticle has a crystallinity value (X_c) of from 20 to 40%, such as from 25 to 35%, such as from 27.5 to 32.4%.

10. The polymeric microparticle according to any one of the preceding clauses, wherein the microparticle has a size of from 10 to less than 100 μm , such as from 30 to 90 μm , such as from 50 to 70 μm .

11. The polymeric microparticle according to any one of the preceding clauses, wherein:

- (a) the polymeric material is a PET and the dye is acridine orange;
- (b) the polymeric material is a PBT and the dye is acridine orange;
- (c) the polymeric material is a PEN and the dye is acridine orange;
- (d) the polymeric material is a PS and the dye is acridine orange;
- (e) the polymeric material is a PE and the dye is acridine orange;
- (f) the polymeric material is a PA and the dye is acridine orange;
- (g) the polymeric material is a PU/PCL blend and the dye is acridine orange; or
- (h) the polymeric material is a PET-PBT blend and the dye is acridine orange;
- (i) the polymeric material is a PET and the dye is methylene blue;
- (j) the polymeric material is a PBT and the dye is methylene blue;
- (k) the polymeric material is a PEN and the dye is methylene blue;
- (l) the polymeric material is a PS and the dye is methylene blue;
- (m) the polymeric material is a PE and the dye is methylene blue;
- (n) the polymeric material is a PA and the dye is methylene blue;
- (o) the polymeric material is a PU/PCL blend and the dye is methylene blue;
- (p) the polymeric material is a PET-PBT blend and the dye is methylene blue;
- (q) the polymeric material is a PET and the dye is toluidine blue O;
- (r) the polymeric material is a PBT and the dye is toluidine blue O;
- (s) the polymeric material is a PEN and the dye is toluidine blue O;
- (t) the polymeric material is a PS and the dye is toluidine blue O;
- (u) the polymeric material is a PE and the dye is toluidine blue O;
- (v) the polymeric material is a PA and the dye is toluidine blue O;
- (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
- (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
- (y) the polymeric material is a PET and the dye is toluidine blue O;
- (z) the polymeric material is a PBT and the dye is toluidine blue O;
- (s) the polymeric material is a PEN and the dye is toluidine blue O;
- (t) the polymeric material is a PS and the dye is toluidine blue O;
- (u) the polymeric material is a PE and the dye is toluidine blue O;
- (v) the polymeric material is a PA and the dye is toluidine blue O;
- (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
- (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;

optionally wherein the polymeric material is selected from the list:

- (i) the polymeric material is a PET and the dye is acridine orange;
- (ii) the polymeric material is a PBT and the dye is acridine orange; or
- (iii) the polymeric material is a PEN and the dye is acridine orange.

12. The polymeric microparticle according to any one of the preceding clauses, wherein the polymeric material is a PET and the dye is acridine orange.

13. A microfluidic device suitable for high throughput identification of one or more of an isolated enzyme, a cell lysate, and a microbe are suitable for degrading a polymeric material, the microfluidic device comprising a plurality of polymeric microparticles as described in any one of Clauses 1 to 12.

14. A method of determining whether one or more of an isolated enzyme, a cell lysate, and a microbe are suitable for degrading a polymeric material, the method comprising the steps of:

- (aa) providing a plurality of polymeric microparticles according to Clauses 1 to 12; and
- (ab) contacting the plurality of polymeric microparticles with the one or more of the isolated enzyme, the cell lysate, or the microbe in a suitable medium for a period of time and measuring the release of the dye over the period of time to determine whether the one or more of the isolated enzyme, the cell lysate, and the microbe are suitable for degrading the polymeric material in the polymeric microparticles.

15. The method according to Clause 14, wherein the method further comprises providing a control comprising a plurality of polymeric microparticles according to Clauses 1 to 12 and a medium so as to provide a background reading over the period of time.

16. The method according to Clause 14 or Clause 15, wherein the dye release is measured using fluorescence.

17. A method of determining whether one or more of an isolated enzyme, a cell lysate, and a microbe are suitable for degrading a polymeric material, the method comprising the steps of:

- (i) providing a microfluidic device according to Clause 13; and
- (ii) contacting the plurality of polymeric microparticles in the microfluidic device with the one or more of the isolated enzyme, the cell lysate, and the microbe in a suitable medium for

a period of time and measuring the release of the dye over the period of time to determine whether the one or more of the isolated enzyme, the cell lysate, and the microbe are suitable for degrading the polymeric material in the polymeric microparticles.

18. A method of making a plurality of polymeric microparticles according to any one of Clauses 1 to 12, the method comprising the steps of:

- (ai) providing a first mixture comprising a polymeric material that is susceptible to enzymatic degradation, a dye and a first solvent, and second mixture comprising a surfactant and an anti-solvent for the polymeric material; and
- (aii) adding the first mixture to an agitated second mixture to provide the plurality of polymeric microparticles, wherein
the dye is soluble in the first solvent and the anti-solvent for the polymeric material.

19. The method according to Clause 18, wherein the first solvent is selected from one or more of the group consisting of toluene, dimethyl formamide, xylene, dichloroacetic acid, and, more particularly chloroform, and hexafluoropropan-2-ol, optionally wherein the first solvent is a mixture of chloroform and hexafluoropropan-2-ol, further optionally wherein the volume to volume ratio of chloroform to hexafluoropropan-2-ol of from 1:1 to 10:1, such as from 2:1 to 5:1, such as about 4:1.

20. The method according to Clause 18 or Clause 19, wherein one or more of the following apply:

- (bi) the anti-solvent is water (e.g. ultra-pure water);
- (bii) the surfactant is a polyvinyl alcohol or poly(vinyl alcohol-co-vinyl acetate) (e.g. a polyvinyl alcohol or poly(vinyl alcohol-co-vinyl acetate) (e.g. 88% vinyl alcohol and 12% vinyl acetate repeating units) with a M_w of about 67,000);
- (biii) the surfactant is present in an amount of from 1 mg surfactant per gram of anti-solvent to 100 mg surfactant per gram of anti-solvent, such as from 10 mg surfactant per gram of anti-solvent to 50 mg surfactant per gram of anti-solvent, such as from 20 mg surfactant per gram of anti-solvent to 30 mg surfactant per gram of anti-solvent.

21. The method according to any one of Clauses 18 to 20, wherein the polymeric material is selected from the group consisting of a polypropylene (PP), a polybutylene adipate terephthalate (PBAT), a polyamide (PA), and more particularly, a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), a polyethylene (PE), a polyurethane (PU) and a polystyrene (PS), copolymers thereof and blends thereof, optionally wherein the polymeric material is selected from the group consisting of a

polyamide (PA), a polystyrene (PS), a polyester-polyether-polyurethane-copolymer (PU/PCL copolymer); and a polyethylene terephthalate and polybutylene terephthalate blend (PET-PBT blend), and, more particularly, a polystyrene (PS), a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), and a polyethylene (PE),

yet more optionally wherein the polymeric material is a PET, a PBT, a PEN, a copolymer thereof, and a blend thereof, optionally wherein the polymeric material is a PET.

22. The method according to any one of Clauses 18 to 21, wherein the dye is a fluorescent dye, optionally wherein the dye is selected from one or more of the group consisting of methylene blue, toluidine blue O, acid fuchsin, alcian blue, sulforhodamine, and more particularly rhodamine B, and acridine orange.

23. The method according to any one of Clauses 18 to 22, wherein the dye is present in the first mixture in a concentration of from 0.001 to 10 mg/mL, such as from 0.005 to 1 mg/mL, such as from 0.01 to 0.05 mg/mL, such as about 0.299 mg/mL.

24. The method according to any one of Clauses 18 to 23, wherein:

- (a) the polymeric material is a PET and the dye is acridine orange;
- (b) the polymeric material is a PBT and the dye is acridine orange;
- (c) the polymeric material is a PEN and the dye is acridine orange;
- (d) the polymeric material is a PS and the dye is acridine orange;
- (e) the polymeric material is a PE and the dye is acridine orange;
- (f) the polymeric material is a PA and the dye is acridine orange;
- (g) the polymeric material is a PU/PCL blend and the dye is acridine orange; or
- (h) the polymeric material is a PET-PBT blend and the dye is acridine orange,
- (i) the polymeric material is a PET and the dye is methylene blue;
- (j) the polymeric material is a PBT and the dye is methylene blue;
- (k) the polymeric material is a PEN and the dye is methylene blue;
- (l) the polymeric material is a PS and the dye is methylene blue;
- (m) the polymeric material is a PE and the dye is methylene blue;
- (n) the polymeric material is a PA and the dye is methylene blue;
- (o) the polymeric material is a PU/PCL blend and the dye is methylene blue;
- (p) the polymeric material is a PET-PBT blend and the dye is methylene blue;
- (q) the polymeric material is a PET and the dye is toluidine blue O;
- (r) the polymeric material is a PBT and the dye is toluidine blue O;
- (s) the polymeric material is a PEN and the dye is toluidine blue O;

- (t) the polymeric material is a PS and the dye is toluidine blue O;
- (u) the polymeric material is a PE and the dye is toluidine blue O;
- (v) the polymeric material is a PA and the dye is toluidine blue O;
- (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
- (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
- (y) the polymeric material is a PET and the dye is toluidine blue O;
- (z) the polymeric material is a PBT and the dye is toluidine blue O;
- (s) the polymeric material is a PEN and the dye is toluidine blue O;
- (t) the polymeric material is a PS and the dye is toluidine blue O;
- (u) the polymeric material is a PE and the dye is toluidine blue O;
- (v) the polymeric material is a PA and the dye is toluidine blue O;
- (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
- (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
optionally wherein the polymeric material is selected from the list:
 - (i) the polymeric material is a PET and the dye is acridine orange;
 - (ii) the polymeric material is a PBT and the dye is acridine orange; or
 - (iii) the polymeric material is a PEN and the dye is acridine orange.

25. The method according to Clause 24, wherein the polymeric material is a PET and the dye is acridine orange.

Drawings

FIG. 1 depicts scanning electron microscopy (SEM) images with magnification of 100 (left), 250 (middle) and 500 (right) of (a) PET, (b) PBT and (c) PEN microspheres containing acridine orange (AO), and (d) flowchart depicting the synthesis of PET microparticles with AO.

FIG. 2 depicts AO standard curve.

FIG. 3 depicts a polymer microparticle-based screening platform for plastic degrading enzymes.

FIG. 4 depicts fluorescence of supernatant from the AO-loaded PET microparticle degradation by WT PETase.

FIG. 5 depicts dye release assay of PET microparticles loaded with different concentration of acridine orange PET microparticles with 1.5 mg, 3 mg and 6 mg of AO per 100 mg PET degradation reaction using WT (wildtype), STAR (STARPETase) and FAST (FASTPETase).

FIG. 6 depicts (a) fluorescence and (b) normalized fluorescence of supernatant from the PBT degradation medium observed after 3 h, 24 h, and 48 h and (b) percentage of PBT degradation observed after 3 h, 24 h, and 48 h.

FIG. 7 depicts (a) fluorescence and (b) normalized fluorescence of supernatant from the PEN degradation medium observed after 3 h, 24 h, and 48 h and (b) percentage of PEN degradation observed after 3 h, 24 h, and 48 h.

FIG. 8 depicts released fluorescence measured after incubation of mPET(AO) beads with *E. coli* cell lysates expressing the indicated enzymes. PyrH: control halogenase enzyme. $n = 3 \pm$ SD.

FIG. 9 depicts the microstructures of (a) PBT, (b) PEN, (c) polymethyl methacrylate (PMMA), and (d) PS polymer microspheres after AO encapsulation.

FIG. 10 depicts (a) SEM image displaying the distribution of the overall microparticle population (left) and high magnification image of microparticle showing partially closed porous morphology (right), (b) dry powder of RhB incorporated microparticles fabricated using the oil-in-water emulsion technique (left) and the excellent redispersion of the powder in aqueous media (right), and (c) histogram plot of the microparticle size distribution.

FIG. 11 depicts (a) the percentage of Rhodamine-B (RhB) adsorbed to the surface of the PET microparticles in the presence and absence of the surfactant SDS, used as a release conditioning agent, (b) the percentage of free RhB retained in the medium when exposed to blank PET particles in the presence of different surfactants (Tween 20, Triton-X 100, and SDS), and (c) the fluorescence intensity of the released RhB from RhB-PET microparticles in media containing NaCl and SDS, as well as media with no additives.

FIG. 12 depicts (a) percentage of change in the mass of the PET powder due to enzymatic degradation in media with and without SDS, (b) HPLC chromatogram displaying the PET degradation monomer peaks, observed only in media without SDS, (c) PETase-mediated PET degradation in media without SDS, with co-present SDS, and post-added SDS (left) and corresponding release of RhB from the degraded microparticles (right).

FIG. 13 depicts (a) percentage degradation of various PET formulations showing notably low degradation of formulations compared to LcPF-Pd, (b) SEM image showing overall particle distribution of various formulations (left), and high magnification image of a microparticle of each formulation (right).

FIG. 14 depicts release of RhB from the microparticles due to the enzymatic degradation of the RhB-PET microparticles by WT and FAST enzyme (a) and the corresponding degradation of polymer measured in the release media (b).

FIG. 15 depicts the microstructures of PET microspheres after encapsulation with (a) acridine orange, (b) Rhodamine B, (c) Rhodamine 101, and (d) sulforhodamine.

Description

It has been surprisingly found that a combination of a dye and a polymeric material in the form of microparticles (the polymeric material encapsulating the dye) can be used to rapidly screen for microbes, cell lysates, or isolated enzymes that are capable of breaking down said polymeric material. In certain embodiments, specific combinations of a dye and a polymeric material may show particular utility in this regard.

Thus, in a first aspect of the invention, there is disclosed a polymeric microparticle comprising:

- a polymeric material that is susceptible to enzymatic degradation; and
- a dye encapsulated within the polymeric material.

For the avoidance of doubt, it will be appreciated that the polymeric material is provided in the form of microparticles that encapsulate the dye.

In embodiments herein, the word “comprising” may be interpreted as requiring the features mentioned, but not limiting the presence of other features. Alternatively, the word “comprising” may also relate to the situation where only the components/features listed are intended to be present (e.g. the word “comprising” may be replaced by the phrases “consists of” or “consists essentially of”). It is explicitly contemplated that both the broader and narrower interpretations can be applied to all aspects and embodiments of the present invention. In other words, the word “comprising” and synonyms thereof may be replaced by the phrase “consisting of” or the phrase “consists essentially of” or synonyms thereof and *vice versa*.

The phrase, "consists essentially of" and its pseudonyms may be interpreted herein to refer to a material where minor impurities may be present. For example, the material may be greater than or equal to 90% pure, such as greater than 95% pure, such as greater than 97% pure, such as greater than 99% pure, such as greater than 99.9% pure, such as greater than 99.99% pure, such as greater than 99.999% pure, such as 100% pure.

As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a composition" includes mixtures of two or more such compositions, reference to "a first solvent" includes mixtures of two or more solvent materials, reference to "the catalyst" includes mixtures of two or more such catalysts, and the like.

The polymeric material in the form of microparticles encapsulates a (e.g. fluorescent) dye. These particles or compartments may be made of various polymeric materials depending on the type of plastic (or polymeric) degrading enzyme that needs to be screened for. During the synthesis of these particles, the dye is encapsulated throughout the polymer. When these dye-encapsulated microparticles are subjected to treatment with the respective degrading enzymes, the encapsulated fluorescent dye is released from the particles which serves as a read out for the activity the degrading enzyme. This significantly reduces the time and processing required to analyze the activity of the degrading enzymes, thus enabling a rapid screening process for identification of novel enzymes and variants.

As will be appreciated therefore, the polymeric microparticles disclosed herein may be used for the rapid identification of novel enzymes and variants (or their producing microbes), and their respective activities, thereby expanding the pool of such enzymes/microbes and enabling new strategies for dealing with plastic/polymeric waste materials.

Given the above, it will be appreciated that the polymeric microparticles disclosed herein will release at least a portion of the dye from the microparticle in an environment containing an enzyme suitable for degrading the polymeric material. This enzyme may be in the form of an isolated enzyme (where a particular enzyme is known or suspected to have suitable degradation properties), a cell lysate or in the form of a microbe (which are suspected of producing such an enzyme). When used herein the term "at least a portion of the dye" is intended to refer to the release of a sufficient amount of the dye from the polymeric material that can be measured by a suitable means or apparatus (this may be, for example, the eye (e.g. with a suitable light source to enable fluorescence to occur), a fluorescence spectrometer or an absorbance spectrometer). These methods may allow for a qualitative and/or a

quantitative reading to be taken for the particular enzyme/microbe expressing an enzyme to be taken.

The polymeric material used in the current invention is not particularly limited, though it should be a material that contains chemical bonds that can be cleaved by an enzyme. It may be, for example, selected from one or more of the group consisting of a polypropylene (PP), a polybutylene adipate terephthalate (PBAT), a polyamide (PA), and more particularly, a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), a polyethylene (PE), a polyurethane (PU) and a polystyrene (PS), copolymers thereof and blends thereof. In more particular embodiment, the polymeric material may be selected from the group consisting of a polyamide (PA), a polystyrene (PS), a polyester-polyether-polyurethane-copolymer (PU/PCL copolymer); and a polyethylene terephthalate and polybutylene terephthalate blend (PET-PBT blend), and, more particularly, a polystyrene (PS), a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), and a polyethylene (PE). For example, the polymeric material may be a PET, a PBT, a PEN, a copolymer thereof, and a blend thereof, optionally wherein the polymeric material may be a PET.

As noted herein in the examples section, the polymeric material may be in the form of copolymers or blends of polymeric materials and these combinations are explicitly intended to be covered by the embodiments discussed in the current application.

While technically any suitable dye may be used, it is particularly preferred that the dye be a fluorescent dye. The combination of the dye and the polymeric material may require tuning to ensure that the dye does not have particularly high binding affinity to the polymeric material it is to be paired with. This may be experimentally determined through the combination of the polymeric material and dye and subjecting the combined material to degradation with an enzyme of known activity for said polymeric material.

Particular fluorescent dyes that may be mentioned herein include, but are not limited to, methylene blue, toluidine blue O, acid fuchsin, alcian blue, sulforhodamine, rhodamine B, acridine orange, and combinations thereof. In yet more particular embodiments of the invention that may be mentioned herein, the dye may be selected from methylene blue, toluidine blue O, acid fuchsin, and acridine orange. In particular embodiments of the invention that may be mentioned herein, the dye may be acridine orange.

It is noted that rhodamine B may not be a good partner in combination with PET, as its binding affinity to this polymeric material may be too high. However, this may be mitigated through the inclusion of additives, such as surfactants. However, it is noted that rhodamine B may be a more suitable combination partner with other polymeric material disclosed herein and may therefore be used as required by the skilled person based on their knowledge of its interaction with a desired polymeric material, either through their knowledge of the field or through a rapid test, as mentioned above.

The amount of the dye within the microparticles may be any sufficient amount to provide a release amount that can be detected by the apparatus being used for that purpose. For example, the dye may be present in an amount of from 0.1 to 10 wt%, such as from 0.5 to 3 wt%, such as from 0.6 to 2 wt%, such as from 0.61 to 1.93 wt%. This weight percent may be relative to the weight of the dye versus the total weight of the polymeric microparticle(s). Note however that the amount of dye is not particularly limited and may depend on the specific dye and polymer(s) to be tested and can be adjusted accordingly by the skilled person following the teachings provided in the current application.

The polymeric microparticles disclosed herein may be partly crystalline. For example, the microparticle may have a crystallinity value (X_c) of from 20 to 40%, such as from 25 to 35%, such as from 27.5 to 32.4%. The crystallinity value may be measured as described in the examples section below.

The polymeric microparticles may have a spherical morphology, though other shapes may be suitable too.

The polymeric microparticles may have a size of from 10 to less than 100 μm , such as from 30 to 90 μm , such as from 50 to 70 μm .

Specific combinations of polymeric materials and dyes that may be mentioned herein include but are not limited to the following list:

- (a) the polymeric material is a PET and the dye is acridine orange;
- (b) the polymeric material is a PBT and the dye is acridine orange;
- (c) the polymeric material is a PEN and the dye is acridine orange;
- (d) the polymeric material is a PS and the dye is acridine orange;
- (e) the polymeric material is a PE and the dye is acridine orange;
- (f) the polymeric material is a PA and the dye is acridine orange;
- (g) the polymeric material is a PET and the dye is methylene blue;

- (h) the polymeric material is a PBT and the dye is methylene blue;
- (i) the polymeric material is a PEN and the dye is methylene blue;
- (j) the polymeric material is a PS and the dye is methylene blue;
- (k) the polymeric material is a PE and the dye is methylene blue;
- (l) the polymeric material is a PA and the dye is methylene blue;
- (m) the polymeric material is a PET and the dye is toluidine blue O;
- (n) the polymeric material is a PBT and the dye is toluidine blue O;
- (o) the polymeric material is a PEN and the dye is toluidine blue O;
- (p) the polymeric material is a PS and the dye is toluidine blue O;
- (q) the polymeric material is a PE and the dye is toluidine blue O;
- (r) the polymeric material is a PA and the dye is toluidine blue O;
- (s) the polymeric material is a PET and the dye is acid fuchsin;
- (t) the polymeric material is a PBT and the dye is acid fuchsin;
- (u) the polymeric material is a PEN and the dye is acid fuchsin;
- (v) the polymeric material is a PS and the dye is acid fuchsin;
- (w) the polymeric material is a PE and the dye is acid fuchsin;
- (x) the polymeric material is a PA and the dye is acid fuchsin;
- (y) the polymeric material is a PU/PCL copolymer and the dye is acridine orange;
- (z) the polymeric material is a PET-PBT blend and the dye is acridine orange;
- (aa) the polymeric material is a PU/PCL copolymer, and the dye is methylene blue;
- (ab) the polymeric material is a PET-PBT blend and the dye is methylene blue;
- (ac) the polymeric material is a PU/PCL copolymer and the dye is toluidine blue O;
- (ad) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
- (ae) the polymeric material is a PU/PCL copolymer and the dye is acid fuchsin; and
- (af) the polymeric material is a PET-PBT blend and the dye is acid fuchsin.

In more particular embodiments, the combination of polymeric materials and dyes that may be mentioned herein include but are not limited to the following list:

- (i) the polymeric material is a PET and the dye is acridine orange;
- (ii) the polymeric material is a PBT and the dye is acridine orange; and
- (iii) the polymeric material is a PEN and the dye is acridine orange.

In yet more particular embodiments, the polymeric material may be a PET and the dye may be acridine orange.

The microparticles disclosed herein may be amendable to handling in such a way that they can be integrated into different products, thereby allowing the formation of useful tools for the detection of enzymes/microbes that can degrade polymeric materials.

Microfluidics are a powerful tool that enables screening of enzyme variants in the millions range. The versatility of the microparticles disclosed herein enables it to be coupled with microfluidics, drastically increasing the potential throughput of the system. Thus, in a further aspect of the invention, there is provided a microfluidic device suitable for high throughput identification of one or more of an isolated enzyme, a cell lysate or a microbe suitable for degrading a polymeric material, the microfluidic device comprising a plurality of polymeric microparticles as described herein. Such a microfluidic device can be designed and operated using the knowledge of the skilled person and the details of the current invention.

Such a microfluidics system and process may entail the production of droplets containing microparticles of the current invention and droplets containing enzymes/microbes, or the droplets produced may contain both. The droplets can then be subjected to sorting and the best performing enzymes/microbes can be isolated (it will be appreciated that the cell lysate's most important component will be the enzyme of interest).

As intimated hereinbefore, the polymeric microparticles mentioned above may be particularly useful for the identification of enzymes/microbes that have the ability to degrade the polymeric material(s) used in the polymeric microparticles. Such methods may also be capable of quantifying the activity of the isolated enzymes, cell lysates or microbes, thereby helping to identify the best-performing enzyme/microbe for use in the degradation of a particular polymeric material.

The terms "microbial" and "microbe" when used herein may refer to any suitable microbial species, such as a bacteria or a yeast or any suitable combination thereof (e.g. two or more bacteria, two or more yeasts, a yeast and a bacteria, two yeasts and a bacteria etc...).

Thus, in a further aspect of the invention, there is provided a method of determining whether an isolated enzyme, a cell lysate or a microbe are suitable for degrading a polymeric material, the method comprising the steps of:

- (aa) providing a plurality of polymeric microparticles as described hereinbefore; and
- (ab) contacting the plurality of polymeric microparticles with the isolated enzyme, the cell lysate or the microbe in a suitable medium for a period of time and measuring the release of

the dye over the period of time to determine whether the enzyme or microbe are suitable for degrading the polymeric material in the polymeric microparticles.

In certain embodiments of this method, a control may be used. That is, the method may further comprise providing a control comprising a plurality of polymeric microparticles as described herein and a medium so as to provide a background reading over the period of time.

In such a method, cell lysates or cells secreting the enzymes can be cultured in multi-well plates containing the dye encapsulated microparticles. Upon degradation of the polymer, the fluorescent dye gets released into the liquid phase which can be readily measured using a plate reader allowing us to identify cells that contain a better degrading enzyme with respect to the WT control. An added advantage with this method is the ability to screen for enzymes at different conditions such as pH and temperature by using cell lysates.

It will be appreciated that any suitable medium (e.g. a liquid medium) that can maintain the biological activity of the enzyme and/or microbe may be used herein and is not particularly limited. Examples of suitable media are provided, but not limited to, those in the examples.

It will be appreciated that the dye release may be measured by any suitable means. This may be completely qualitative (e.g. by eye detecting fluorescence) or it may be measured in a way that may be somewhat more quantitative (e.g. one or both of fluorescence and absorbance (e.g. fluorescence) as determined with a suitable spectrometer). It will be appreciated that these detection means may be used in the other methods discussed hereinbelow too.

In a further aspect of the invention, there is provided a method of determining whether an isolated enzyme, a cell lysate or a microbe are suitable for degrading a polymeric material, the method comprising the steps of:

- (i) providing a microfluidic device as discussed herein; and
- (ii) contacting the plurality of polymeric microparticles in the microfluidic device with the isolated enzyme, a cell lysate or the microbe in a suitable medium for a period of time and measuring the release of the dye over the period of time to determine whether the enzyme or bacteria are suitable for degrading the polymeric material in the polymeric microparticles.

In a further aspect of the invention there is provided a method of making a plurality of polymeric microparticles as described hereinbefore, the method comprising the steps of:

- (ai) providing a first mixture comprising a polymeric material that is susceptible to enzymatic degradation, a dye and a first solvent, and second mixture comprising a surfactant and an anti-solvent for the polymeric material; and
- (aii) adding the first mixture to an agitated second mixture to provide the plurality of polymeric microparticles, wherein
the dye is soluble in the first solvent and the anti-solvent for the polymeric material.

As will be appreciated, the first solvent may be a single solvent or it may be a mixture of different solvents to achieve the desired formation of the microparticles. To that end, it may be desired that the solvent and anti-solvent are immiscible together, or are miscible together. The selection of the solvents may ultimately depend on the polymer(s) being tested and these can be selected accordingly. It will be appreciated that the first solvent should be able to solubilise the polymeric material and the dye. In certain embodiments that may be mentioned herein, the first solvent may be selected from one or more of the group consisting of toluene, dimethyl formamide, xylene, dichloroacetic acid, and, more particularly chloroform, and hexafluoropropan-2-ol, optionally wherein the first solvent is hexafluoropropan-2-ol. As an example of a first solvent mixture, the first solvent may be formed from chloroform and hexafluoropropan-2-ol in any suitable ratio.

For example, the first solvent may be selected from one or more of the group consisting of toluene, dimethyl formamide, xylene, dichloroacetic acid, and, more particularly chloroform, and hexafluoropropan-2-ol, optionally wherein the first solvent is a mixture of chloroform and hexafluoropropan-2-ol. In embodiments wherein chloroform and hexafluoropropan-2-ol are used together as the first solvent, any suitable volume to volume ratio may be used. For example, the volume to volume ratio of chloroform to hexafluoropropan-2-ol may be from 1:1 to 10:1, such as from 2:1 to 5:1, such as about 4:1.

The anti-solvent used in the method of manufacture may be any suitable material that causes precipitation of the polymer from solution. For example, the anti-solvent may be water (e.g. ultra-pure water).

Any suitable surfactant may be used herein. For example, the surfactant may be a polyvinyl alcohol or poly(vinyl alcohol-co-vinyl acetate) (e.g. a polyvinyl alcohol or poly(vinyl alcohol-co-vinyl acetate) (e.g. 88% vinyl alcohol and 12% vinyl acetate repeating units) with a M_w of about 67,000). The surfactant may be used in any suitable amount. For example, the surfactant may be present in an amount of from 1 mg surfactant per gram of anti-solvent to 100 mg surfactant per gram of anti-solvent, such as from 10 mg surfactant per gram of anti-solvent to 50 mg

surfactant per gram of anti-solvent, such as from 20 mg surfactant per gram of anti-solvent to 30 mg surfactant per gram of anti-solvent. For example a suitable surfactant that may be mentioned in the examples is Mowiol™ 8-88, which is a poly(vinyl alcohol-co-vinyl acetate) having 88% vinyl alcohol and 12% vinyl acetate repeating units and a M_w of around 67,000 Daltons.

The polymeric material used herein is not particularly limited. As will be appreciated, the list of polymeric materials discussed above for the polymeric microparticles *per se* may be used. For the sake of brevity, this list is not repeated here.

The dye used in the method may be chosen from the dyes mentioned hereinbefore in relation to the polymeric microparticles *per se*. Again, this list is not repeated here for the sake of brevity.

The dye may be present in any suitable amount in the first mixture. For example, the dye may be present in the first mixture in a concentration of from 0.001 to 10 mg/mL, such as from 0.005 to 1 mg/mL, such as from 0.01 to 0.05 mg/mL, such as about 0.299 mg/mL. In certain embodiments, it is believed that the loading efficiency of the dye may be from 0.01% to 5%.

In certain embodiments of the method, that may be mentioned herein:

- (a) the polymeric material is a PET and the dye is acridine orange;
- (b) the polymeric material is a PBT and the dye is acridine orange;
- (c) the polymeric material is a PEN and the dye is acridine orange;
- (d) the polymeric material is a PS and the dye is acridine orange;
- (e) the polymeric material is a PE and the dye is acridine orange;
- (f) the polymeric material is a PA and the dye is acridine orange;
- (g) the polymeric material is a PET and the dye is methylene blue;
- (h) the polymeric material is a PBT and the dye is methylene blue;
- (i) the polymeric material is a PEN and the dye is methylene blue;
- (j) the polymeric material is a PS and the dye is methylene blue;
- (k) the polymeric material is a PE and the dye is methylene blue;
- (l) the polymeric material is a PA and the dye is methylene blue;
- (m) the polymeric material is a PET and the dye is toluidine blue O;
- (n) the polymeric material is a PBT and the dye is toluidine blue O;
- (o) the polymeric material is a PEN and the dye is toluidine blue O;
- (p) the polymeric material is a PS and the dye is toluidine blue O;
- (q) the polymeric material is a PE and the dye is toluidine blue O;

- (r) the polymeric material is a PA and the dye is toluidine blue O;
- (s) the polymeric material is a PET and the dye is acid fuchsin;
- (t) the polymeric material is a PBT and the dye is acid fuchsin;
- (u) the polymeric material is a PEN and the dye is acid fuchsin;
- (v) the polymeric material is a PS and the dye is acid fuchsin;
- (w) the polymeric material is a PE and the dye is acid fuchsin;
- (x) the polymeric material is a PA and the dye is acid fuchsin;
- (y) the polymeric material is a PU/PCL copolymer and the dye is acridine orange;
- (z) the polymeric material is a PET-PBT blend and the dye is acridine orange;
- (aa) the polymeric material is a PU/PCL copolymer, and the dye is methylene blue;
- (ab) the polymeric material is a PET-PBT blend and the dye is methylene blue;
- (ac) the polymeric material is a PU/PCL copolymer and the dye is toluidine blue O;
- (ad) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
- (ae) the polymeric material is a PU/PCL copolymer and the dye is acid fuchsin; and
- (af) the polymeric material is a PET-PBT blend and the dye is acid fuchsin.

For example:

- (i) the polymeric material is a PET and the dye is acridine orange;
- (ii) the polymeric material is a PBT and the dye is acridine orange; or
- (iii) the polymeric material is a PEN and the dye is acridine orange.

In yet more particular embodiments the polymeric material may be a PET and the dye may be acridine orange.

- Our method describes the screening of plastic degrading enzymes using polymer microparticles for the identification of enzymes with enhanced activity.
- Our method is versatile and can be adapted to cytosolic and secretory expression of plastic degrading enzymes while also allowing them to be used for screening in both solid and liquid phase screening methods.
- This method eliminates the use culturing and purification of enzymes. Cell lysates or enzymes secreted from cells can be used directly in the assay drastically reducing the number of steps required for screening.
- Unlike existing methods that use proxy substrates such as pNPA , our platform uses real plastic substrate in the form of microparticles which helps identify the enzymes that are able to degrade the polymer structure in real case scenarios.

The current methods are extremely labor intensive and require sample preparation and analysis steps that require several minutes to analyze a single sample. The disclosed products and methods are very efficient as allow for measurements in less than a minute for up to 96 samples, or even more. Conventional prior art fluorescent methods require co-hydrolysis of the substrate as well as the fluorescent moiety where parameters such as pH and temperature can affect the co-hydrolysis resulting in unreliable readouts. The currently disclosed method can be easily adapted for various scenarios, such as pH, temperature, time of analysis and can be performed in solid or liquid phase and can employ cytosolic or secretory expression of PET hydrolases (or similar enzymes for other polymeric materials). Moreover, this platform is not limited to the identification of just PET hydrolases but can be employed to other plastic polymers such as PET, PBT, PEN, PU, and PS among others, as mentioned hereinbefore (and as exemplified in the examples).

While some reported methods use molecules that are similar to PET polymer (e.g., containing ester bonds) referred to as a proxy substrate, they fail to show that the activity results are translatable to a real PET substrate. That is, if high activity is detected on proxy substrate, it does not necessarily mean that it will have high activity on the real PET substrate. In fact, we have observed the reverse.

The disclosed method is as easy as adding the dye-loaded polymer (e.g. mPET) into the reaction mixture and let the reaction run for a pre-determined time. At the end of the reaction time, the reaction mixture is spun to remove any debris and the released dye will be measured using a spectrophotometer. In contrast, some of the current methods will require an additional reagent (e.g., dye) as a reporter/indicator.

The disclosed microparticles provide a screening platform that is a powerful tool for the identification of improved and efficient plastic degrading enzymes. It may find application as an assay following random mutagenesis or directed evolution strategies that result in vast sets of plastic degrading enzyme mutants. Thus the disclosed platform may aid in the rapid identification of mutants with superior performance which is currently only limited by availability of screening tools.

With the discovery of enzymes such as PETase that have the ability to degrade PET plastics, the focus is quickly shifting towards development of processes for biocatalysts of plastic wastes as an alternate route to recycling. Platforms such as the ones disclosed herein are key to attaining these goals where the first step is the identification of best performing enzymes.

These platforms are may be important for establishing commercial scale development of plastic waste recycling strategies.

Certain aspects of the invention that may be mentioned herein relate to the following numbered statements.

1. A microparticle comprising:
 - a) A polymer that may be degraded by enzymes; and
 - b) A dye encapsulated within the polymer.
2. The microparticle according to Statement 1, wherein the polymer is polyethylene terephthalate (PET), polybutylene terephthalate (PBT) or polyethylene naphthalate (PEN).
3. The microparticle according to Statement 1 or 2, wherein the dye is acridine orange, Rhodamine B or Rhodamine 101.

The microparticles may be synthesized using an emulsion/solvent evaporation method.

Further aspects and embodiments of the invention will now be discussed by reference to the following non-limiting examples.

Examples

Materials

Polyethylene terephthalate film (PET film, GF59152350), polyethylene naphthalate film (PEN film, GF80046851) and polybutylene terephthalate film (PBT film, GF90577468), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, $\geq 99\%$), dichloroacetic acid ($\geq 99\%$), Mowiol 8-88 (MW $\approx 67,000$ g.mol⁻¹), formic acid (reagent grade, $\geq 95\%$), ethylene glycol anhydrous (99.8%), 1,4-butanediol (99%), terephthaloyl chloride (TPCI, $\geq 99\%$), 2-bromoethanol (95%), sodium hydrogen carbonate (99.7 %), dimethyl formamide (anhydrous, 99.7%), potassium hydroxide (90 %), thionyl chloride (99 %), sodium hydroxide (97 %), magnesium sulphate (anhydrous 99.5 %), sodium sulphate (99 %) and dimethyl sufoxide (DMSO, $\geq 99\%$), Poly(methyl metacrylate), polystyrene (MW = 35,000) were provided by Sigma-Aldrich. Acetonitrile (HPLC/Spectro) and chloroform (stabilized) (HPLC/Spectro) were obtained from Tedia. TPA, MHET, BHET and naphthalene-2,6-dicarboxylic acid (NDA), acridine orange hemi(zinc

chloride) salt (dye content 85%), sulforhodamine B (dye content 75 %) and rhodamine 101 inner salt are from Merk KGaA. SDS is from Hoefer Inc. Chloridric acid (37 %) is from supelco.

Analytical techniques

SEM

The sample powder was dispersed in water (5×10^{-1} mg mL⁻¹). The dispersion was drop-casted on aluminium, air dried, and was subsequently coated with an ultrathin Pt layer to minimize charging. The SEM images were obtained using a Schottky field emission scanning electron microscope (JEOL JSM-7800FPRIME), at an acceleration voltage of 5 kV. Magnifications are specified in pictures captions.

HPLC

The samples were analysed using 1260 Infinity HPLC (Agilent Technology) connected to a UV/Vis detector (G1314A WD) and C18 column (Eclipse Plus C18, 5 μ m, 4.6 x 150 mm) was used. Mobile phase A was water containing 1% formic acid and mobile phase B was acetonitrile and the flow rate was fixed at 1 mL.min⁻¹. The mobile phase was changed gradually from 5 % to 25 % mobile phase A over 15 min, remained at 25 % mobile phase A for 2 min, 25 % to 5 % mobile phase A for 1 min, and remained at 5 % mobile phase A for 5 min. The detection wavelength was set at 260 nm. All experiments were performed in triplicate. Samples consisted of 50 μ L of supernatant and 50 μ L of DMSO.

Example 1. Development of dye encapsulated polymer particles (mPET(AO), mPBT(AO) and mPEN(AO))

To address the challenge of screening libraries of plastic degrading enzymes, the development of micro compartments that encapsulate fluorescent dye was envisioned. These compartments or particles may be made of various plastic polymers depending on the type of plastic degrading enzyme that needs to be screened for. During the synthesis of these particles, fluorescent dyes are encapsulated throughout the polymer. When these dye encapsulated particles are subjected to treatment with the respective degrading enzymes, the encapsulated fluorescent dye is released into the supernatant, the fluorescence of which can be readily measured in a plate reader. This significantly reduces the time and processing required to analyze the activity of the degrading enzymes thus, enabling a rapid screening process for identification of novel enzymes and variants.

To be able to screen the enzymes, a fluorescent dye, AO was encapsulated in polymer microspheres. This was realized with an emulsion/solvent evaporation method, as described below.

Synthesis of plastic microparticles – PET microparticles (mPET(AO))

100 mg of PET were dissolved in 10 mL of chloroform/hexafluoropropan-2-ol (HFIP) 8/2 v/v. Acridine orange (AO) solution was prepared by dissolving AO in HFIP to obtain a concentration of 100 mg.mL⁻¹. 30 µL of AO solution were added to the PET solution. This solution was then sonicated for 30 s with a sonication probe. Separately, 4 g of Mowiol 8-88 were dissolved into 200 mL of miliQ water. The water containing surfactant was stirred at 400 rpm using a mechanical stirrer. The polymer solution was slowly poured into the water solution and the emulsion was left overnight under stirring. Then, the mixture was passed through a sieve to remove all the particles whose diameter exceeds 71 µm. The particles were then washed 5 to 8 times and freeze dried overnight. The obtained particles were called mPET(AO).

As shown in FIG. 1d, the method for synthesis of mPET(AO) 100 includes dissolution of PET and AO in HFIP (3 mg of AO/100mg of PET) 110, homogenization and resuspension in water 120, solvent evaporation 130, and wash step and freeze drying of particles 140.

Synthesis of plastic microparticles – PBT microparticles (mPBT(AO))

PBT microparticles were synthesized by following the procedure for PET microparticles except PBT was used.

Synthesis of plastic microparticles – PEN microparticles (mPEN(AO))

PEN microparticles were synthesized by following the procedure for PET microparticles except PEN was used and an additional heating step for dissolution of PET in HFIP/dichloroacetic acid 1/1 v/v was included.

100 mg of PEN was dissolved in 2 mL of HFIP/dichloroacetic acid 1/1 v/v and heated at 65 °C. 8 mL of chloroform was added to the 2 mL of PEN solution. 2 g of Mowiol 8-88 was dissolved in 200 mL water. The polymer solution is added slowly into the solution of water and Mowiol 8-88, under mechanical stirring (400 rpm). The emulsion was left under stirring overnight. Filtration and purification steps were as in PET microsphere synthesis.

Synthesis of PS microparticles (mPS(AO))

100 mg of PS was dissolved in 10 mL of CHCl₃ (PS Solution). 5 mg of AO was dissolved in 3 mL of HFIP (AO solution). Then, 100 µL of AO solution was added to the PS solution. The

polymer solution was slowly poured into 200 mL of DI water containing 4 g of Mowiol 8-88. The emulsion was stirred at 400 rpm for 24 h. Filtration and washing steps were same as for PET.

Synthesis of PMMA microparticles (mPMMA(AO))

The synthesis of mPMMA(AO) was carried out by following the protocol for synthesizing mPS(AO) except PMMA was used.

Example 2. Characterization of dye encapsulated polymer particles (mPET(AO), mPBT(AO) and mPEN(AO))

Differential scanning calorimetry (DSC)

All materials were characterized by DSC. Crystallinity was calculated as follows:

$$X_c(\%) = \frac{100 \times (\Delta H_m - \Delta H_{cc})}{\Delta H_m(100\%)}$$

where ΔH_m (100%) is enthalpy of melting for a fully crystalline polymer.

Loading efficiency

Loading efficiency was measured with calibration curves. Standards were prepared by dissolving different amount of dye in HFIP. Then, 1 mg of embedded polymer was also dissolved in HFIP. Absorbance were measured. Then, the amount of dye in the microspheres was calculated from the calibration curve and the loading efficiency was deducted.

Results and discussion

FIG. 1 depicts the SEM images of mPET(AO), mPBT(AO) and mPEN(AO). Loading efficiency of particles was measured using the absorbance of AO at 487 nm. A calibration curve was drawn (FIG. 2). Then, microspheres were dissolved into HFIP and absorbance was measured; values are provided in Table 1.

Table 1. Loading efficiency of AO in polymer microspheres.

Polymer used	AO loading efficiency
PET	65.7 %
PBT	20.5 %
PEN	31.5 %

Table 2. Thermal parameters and crystallinity of different materials.

Material	T _m (°C)	ΔH _m (J.g ⁻¹)	T _{mc} (°C)	ΔH _{mc} (J.g ⁻¹)	Xc (%)
PET film					
mPET(AO)	246	45	166	38	32.0
PBT film	223	48	191	55	33.8
mPBT(AO)	223	46	195	38	32.4
PEN film	269	40.5	-	-	28.5
mPEN(AO)	268	39	-	-	27.5

Example 3. Dye release assay using synthesized microparticles (mPET(AO), mPBT(AO) and mPEN(AO))

Dye release assay

Shuffle cells expressing PET-degrading or control enzymes were induced overnight in Terrific Broth and 1 mL of the culture was spun down, lysed in lysis buffer containing 100 mM sodium phosphate (pH = 8.0) with 1 mg/mL lysozyme at 37 °C. The samples were spun down and 100 μL of the supernatant incubated with 100 μL of 100 mM sodium phosphate (pH = 8.0) containing 1 mg of mPET(AO) beads overnight at the optimal temperatures specific for each enzyme. The reactions were spun down for 5 minutes at 4,000 x g and 100 μL of the supernatant was taken for fluorescence measuring using Envision plate reader 490-520 nm. The same protocol was used to study mPBT(AO) and mPEN(AO) with IsPETase and FAST PETase.

As shown in FIG. 3, there is a polymer microparticle-based screening platform for plastic degrading enzymes 300 that includes the following steps:

310. synthetic polymer are prepared in the form of microparticles loaded with dye;
320. microparticles are suspended in media with mutants. By enzyme catalysis, microparticles degrade and release dye in the media; and
330. fluorescence of dye release is measured to identify mutants with higher activity.

Results and discussion

First, polymer microspheres were incubated at 30 °C for 30 minutes and centrifuged to remove all the leaking dye. The synthesized mPET(AO) were subjected to degradation by PETase. After the incubation period, fluorescence recorded at 1 hour, 6 hours, 24 hours and 48 hours using a plate reader at $\lambda_{\text{excitation}} = 495 \text{ nm}$ and $\lambda_{\text{emission}} = 520 \text{ nm}$ shows a trend of increase in fluorescence, validating the progress of reaction catalyzed by PETase, as shown in FIG. 4. The particles show a stable dye release in the absence of enzyme due to leaching that is

considered as background reading and can be used to normalize the reading of test reaction. The trend of increase in fluorescence reading with increase in activity was observed.

The trend of increase in fluorescence reading with increase in activity was also observed with enzyme variants that are reported to show enhanced activity over WT-PETase, FAST-PETase and STAR-PETase. The dye release trends with WT-, STAR- and FAST-PETase are shown to co-relate with the amount of terephthalic acid released due to degradation after 6 hours of reaction, as shown in FIG. 5.

The dye release assay was performed using mPEN(AO) and mPBT(AO), which shows an increase in fluorescence reading, as the reaction progresses. The dye released by mPBT(AO) and mPEN(AO) (FIGS. 6b and 7b) was observed following the trend in increasing percent degradation analyzed by HPLC method (FIGS. 6c and 7c).

Therefore, this system is efficient to screen enzymes and can work for several semi-aromatic polyesters.

Next, the mPET(AO) particles were tested using *E. coli* cell lysates expressing PET-degrading or control enzymes. The results show clear release of AO dye in the presence of WT-PETase and FAST-PETase but not control enzyme (PyrH) containing lysate (FIG. 8). Furthermore, the activity of FAST-PETase is clearly superior, commensurate with the improved activity reported for this variant.

Table 3. Encapsulation of AO in other polymers. FIG. 9 depicts the microstructures of polymer microspheres after AO encapsulation.

Polymer	Encapsulation of AO	Loading efficiency	Correlation between Fluorescence and degradation
PBT	Yes	20.5 %	Good
PEN	Yes	31.5 %	Good
PMMA	Yes	12.2 %	Not Tested
PS	Yes	3.0 %	Not Tested

Comparative Example 1

Table 4. Comparison of mPET(AO), mPBT(AO) and mPEN(AO) with recently reported screening methods.

Comparison with recently reported screening methods				
Parameters	Fe ²⁺ reagent-based screening (Zurier, H. S. & Goddard, J. M., Biotechnol. Bioeng. 2023, 120 (4), 1000-1014)	BHET-OH based screening (Shi, L. X. et al., Angew. Chem. 2023, 135, e202218390	FP co hydrolysis conjugated screening (Liu, K. et al., ChemSusChem 2023, 16, e202202019)	Microparticle based (The present disclosure)
Substrate used	PET microfibre	BHET (Proxy substrate)	PET microfibre embedded on well plate	Polymer microparticles – mPET(AO), mPBT(AO) and mPEN(AO) (high crystalline)
Mechanism	Fenton reaction conjugated (fluorescent by-product detection)	Fenton reaction conjugated (TPA-OH)	FDL co hydrolysis – Fluorescein detection	Dye release upon degradation
Detected species	Fluorescence of TPA-OH	Fluorescence of TPA-OH	Fluorescein	Dye released – AO
Number of steps	4	3	4	3
pH and temperature control	No	No	No	Yes

Example 4. Microfluidics based high throughput screening using mPET

Microfluidics is a powerful tool that enables screening of enzyme variants in the millions range. The versatility of our platforms enables it to be coupled with microfluidics drastically exemplifying the scale. This process entails production of droplets containing mPET encapsulating reporters (e.g. fluorescent dyes) and bacteria producing PETase mutants (secreting or otherwise). The droplets will be subjected to sorting and the best performing mutants under specified parameters to be determined in the future will be isolated. This can be extrapolated likewise to other polymers and enzymes that degrade them.

Example 5. Engineering of Rhodamine-B (RhB) dye loaded microparticle system of PET as a screening platform for enzyme

Rhodamine-B (RhB) incorporated microparticles of PET (RhB-MPs) were fabricated using an emulsion solvent evaporation technique with commercially purchased PET film. This is a simple, scalable, and facile method that allows particle size distribution control. The method entails dissolving a polymer in a volatile organic solvent (oil phase) that is immiscible with water. A dye is dispersed in this polymer solution, which is then emulsified in an aqueous solution that contains a surfactant (water phase) to produce an oil-in-water emulsion, which forms discrete droplets. The organic solvent is then removed by evaporation while continuously stirring, resulting in free-flowing solid microparticles of the polymer after appropriate filtration and drying.

The success of this technique depends largely on the choice of solvent. The volatile solvent must be able to dissolve the polymer completely and is immiscible with the aqueous phase. It is challenging to completely dissolve PET in most common solvents such as orthochlorophenol and phenol/tetrachloroethane. These solvents dissolve PET only at high temperature, which can inadvertently degrade the polymer in fabrication process. If there is any degradation during the preparation of the microparticles, the accuracy of the screening platform to accurately assess the effectiveness of PET degrading enzymes may be affected. HFIP was chosen as the solvent due to its capability of dissolving the PET at room temperature with no signs of polymer degradation.

HFIP is miscible with water and thus it is impossible to form an emulsion in aqueous phase. As such, another water immiscible solvent, chloroform, was used as a co-solvent with HFIP at a volume ratio of 1:4 imparting immiscibility to the resultant polymer solution with the aqueous phase. This ratio was determined experimentally to ensure that the PET remains in solution

without precipitation in the organic solvent mixture while maintaining immiscibility with the aqueous phase. HFIP-PET solution and chloroform did not naturally blend with each other and required ultrasonication to form a clear homogeneous solution which was then quickly emulsified with the aqueous phase to form emulsion droplets. Due to the solubility differences between the two solvents - HFIP and chloroform, they tend to phase separate within each of the emulsion droplets during the solvent evaporation process promoting polymer precipitation at different rates.

Synthesis of RhB-MPs

Stock solutions of Rhodamine-B (RhB) and polyethylene terephthalate (PET) in chloroform, with concentrations of 16.67 mg/ml and 50 mg/ml, respectively were prepared. Subsequently, we combined 10 μ l of the RhB solution with 2 ml of the PET polymer solution. To this mixture, 8 ml of chloroform was added and thoroughly blended using an ultrasound probe at 50 amplitude for 60 seconds. The resulting solution, now containing RhB dye, was swiftly emulsified in a 200 ml aqueous solution of polyvinyl alcohol (1%w/v), utilizing an overhead stirrer operating at 400 rpm. This emulsion constituted an oil-in-water emulsion. The organic solvents were then methodically evaporated with continuous stirring over a period of six hours, leading to the formation of solid PET microparticles incorporating RhB. To ensure size uniformity, these microparticles were sieved through a 70 μ m sieve, meticulously washed three times with deionized water, and subsequently freeze-dried overnight, yielding finely dispersed and free-flowing PET microparticles encapsulating RhB.

Example 6. Characterization of RhB-MPs

Encapsulation efficiency and Loading capacity

1 mg of the microparticles were dissolved in 1 mL HFIP which effectively dissolves PET and releases the encapsulated dye. After centrifugation to remove any undissolved debris, the concentration of RhB in the supernatant was determined using a standard curve. The standard curve was generated by plotting the fluorescence intensity of known concentrations of RhB in HFIP against their respective concentrations.

Encapsulation efficiency (EE) was calculated using the formula:

$$EE (\%) = \frac{\text{amount of encapsulated RhB}}{\text{Total amount of RhB used}} \times 100$$

Loading capacity (LC) was determined as the amount of encapsulated dye relative to the total mass of microparticles using the formula:

$$LC (\%) = \frac{\text{amount of encapsulated RhB}}{\text{total mass of microparticles}} \times 100$$

Results and discussion

Spherical microparticles with partially closed surface morphology are produced instead of smooth closed surface (FIG. 10a). This, however, is a desirable outcome as the porosity and imperfection observed in the surface of the microparticles may allow for faster degradation as the enzyme solution can better penetrate the microparticles.

The study resulted in the successful fabrication of free-flowing PET microparticles with high dye loading, as evidenced by the pink color imparted to the microparticles (FIG. 10b). The encapsulation efficiency, loading capacity, and yield of the microparticles were determined to be $94.1 \pm 3.8\%$, $0.017 \pm 0.001\%$, and $75.0 \pm 4.0\%$, respectively. Upon redispersion in aqueous phase, the microparticles exhibited excellent dispersibility. The size distribution of the microparticles was plotted as a histogram and was found to follow a Gaussian distribution. The average size of the microparticles was determined to be $47.9 \pm 10.3\mu\text{m}$, with D(90) and D(10) values of $63\mu\text{m}$ and $36\mu\text{m}$, respectively (FIG. 10c).

Example 7. Effect of salts and surfactants on mitigating dye-polymer adsorption and enhancing release for enzymatic activity assessment

Enzymatic activity assessment

1. Effect of SDS on mitigation of dye-polymer adsorption

Specifically, two separate tubes were prepared. In each tube, 1 mg of LCPF-Pd was suspended in 250 μL of media containing Rhodamine B (RhB) at a concentration of 0.67 $\mu\text{g}/\text{mL}$. SDS was introduced at a concentration of 0.25% w/v in one tube, while the other tube served as the control without SDS. Both tubes were placed on a thermomixer operating at 1000 rpm at room temperature, and samples were collected at 0.5 hours and 24 hours to measure the fluorescence intensity of RhB. The percentage of adsorbed RhB, calculated as the difference in fluorescence intensity between the sample tube and the control at these specified time intervals, was subsequently plotted. Similar experiments were conducted with Triton X-100 and Tween 20 for comparison.

2. Effect of SDS on release of RhB upon degradation of particles by enzymatic activity

1 mg of dye-loaded microparticles was suspended in 250 μL of reaction mixture under three conditions: (1) with PETase enzyme and SDS, (2) with PETase enzyme and NaCl, and (3) with PETase enzyme alone as a control. These were placed on a thermomixer operating at

1000 rpm at room temperature. At predetermined time points i.e, 0.5 hours, 1 hour and 24 hours the samples were centrifuged and clear supernatant was collected to measure the fluorescence intensity of RhB and generate the plot.

Results and discussion

RhB is a fluorescent dye with a high molar absorption coefficient and quantum yield, making it an excellent probe for fluorescence-based assays. It has been reported to have a strong natural binding affinity with the PET and thus allows for facile encapsulation in PET microparticles. However, when these fabricated RhB-MPs were degraded overnight in the media containing PETase enzyme, the dye was not detected in the media, possibly because of the dye not being detached from the degraded polymer due to strong binding affinity.

In the present disclosure, when blank PET powder (LcPF-Pd: prepared by cryogenic grinding PET film) was suspended in RhB solution, the concentration of RhB in the solution dropped by 40% in 30 minutes, subsequently reaching 65% in 24 hours (FIG. 11a). This decrease in the concentration of free Rh-B in the solution suggests that the dye had adsorbed to the surface of the polymer. It is critical to mitigate this issue so that the dye released from the microparticles as the enzymes degrade them can be accurately measured and correlated to the enzymatic degradation.

The efficacy of three surfactants sodium dodecyl sulfate (SDS), Tween 20 and Triton x-100 was assessed. It was observed that SDS consistently was the most effective in preventing the adsorption of dye over the 24-hr. study duration (FIG. 11b). Contrary to 65% of dye loss from the free solution due to adsorption, the loss was less than 5% in 24 hours when 0.25% SDS was added to the media (FIG. 11a). It was evident that the SDS, added at an amount just above its critical micellar concentration (cmc) was effective in mitigating the binding issue. The observation was further corroborated in our study where the RhB-PET were analyzed for release in the media containing PETase enzyme: 1. with SDS, and 2. without SDS (FIG. 11c). A significant increase in the fluorescence signal intensity of the RhB is seen in the presence of SDS. While the fluorescence of the samples without SDS remained consistently less than 25 units over the 24 hours study period, a significantly higher fluorescence signal of RhB was detected in the presence of SDS, starting from 180 units at 30 mins, and increasing gradually over time to reach 325 units at 24 hours. Since the PETase was present in both the samples, it can be deduced that the inclusion of SDS helped in facilitating the release of RhB from the degraded polymer matrix by disrupting the binding affinity and retaining the released RhB in the media by favorable interaction with the released RhB molecule. NaCl, as an alternative to SDS, was however found to be ineffective in retaining the released dye in the media. The

fluorescence intensity of the sample with NaCl was observed to be equally low as the sample without any release conditioners. Hence, SDS was finalized as the release conditioner in further experiments.

Example 8. Surfactant concentration and its effects on polymer degradation and dye release

Polymer degradation and dye release study

1 mg of dye-loaded microparticles was suspended in 250 μ L of reaction mixture containing PETase (10 μ M) enzyme under three conditions: (1) with concurrently added SDS, (2) with SDS added at the end of the reaction duration (24 h), and (3) with PETase enzyme alone as a control. These were placed on a thermomixer operating at 1000 rpm at room temperature for 24 h, centrifuged and clear supernatant were collected to measure the fluorescence intensity of released RhB and generate the plot. The samples were also tested with HPLC to analyze the PET degradation products.

Results and discussion

While SDS was conducive in mitigating the dye-polymer binding issue, we found that the usage of SDS hindered the enzymatic degradation of PET as the monomers of PET could not be detected in HPLC analysis of the corresponding samples discussed in FIG. 11c. This was also evident from the residual dry weight measurements of LcPF-PD suspended in media containing PETase enzyme. Specifically, the residual dry weight of the LcPF-PD decreased by 6.6% when SDS was absent, while it increased by 0.5% for samples with SDS present (FIG. 12a). The sample's slight increase in mass may be due to the residual solutes in the media, which remain in the tube after drying the samples for measurement as this is also observed in the control tubes with blank media with and without SDS. The results were corroborated by analyzing the sample media using HPLC to monitor the monomers of PET. Two peaks, corresponding to terephthalic acid (TPA) and mono(2-hydroxyethyl) terephthalic acid (MHET), were detected for the sample without SDS eluting at 10.5 min and 13.5 min respectively, while no such peaks were observed in the sample with SDS and control samples (FIG. 12b). This confirmed that SDS prevented the degradation of the PET. The increased fluorescence in release media containing SDS observed in FIG. 11c, thus could not be solely attributed to the particle degradation causing the release of dye. Rather, this could be because of surface bound RhB being extracted from the microparticles and retained by SDS in the release media.

Contrary to our observation, previous studies have shown that anionic and cationic surfactants can accelerate the degradation of PET by facilitating enzymes of opposite charge to the polymer surface. For instance, Makoto Furukawa et al. found that pre-incubating in SDS at its optimal concentration (0.025% w/v) dramatically increased initial 3-hour catalytic activity from 0.07 to 3.2 nmol min⁻¹ cm² (M. Furukawa *et al.*, *Sci. Rep.* **2019**, 9). This was attributed to the anionic SDS molecule coating the polymer surface and attracting the cationic enzyme resulting in accelerated interaction. Further increasing SDS concentration to 0.04%, however, did not accelerate degradation at all, suggesting that at the 0.25% concentration used in the present disclosure (10 times higher than the reported optimal concentration), SDS prevents polymer degradation possibly by densely packing onto the polymer surface, rendering it inaccessible to the enzyme. But, decreasing the concentration of SDS to 0.025% was not feasible in the present disclosure, as SDS was effective in mitigating the RhB -polymer binding only above 0.25%, which is slightly above its cmc value.

These observations led us to modifying the protocol of degradation study. The RhB-MPs would firstly be exposed to the enzymes in the absence of SDS thereby allowing the enzyme to degrade the polymer up to the predetermined time points without any unfavorable interference of SDS. Subsequently, SDS would be added to the sample tubes and agitated briefly so that the SDS completely retrieves the RhB from the degraded MPs by disrupting the dye-polymer binding. We assessed the feasibility of this approach by comparing the released amount of RhB and polymer monomers in three different scenarios: without SDS, with co-present SDS, and with post-added SDS. We found that without surfactant, the UV absorbance intensity attributed to the monomers of PET degradation was 7-fold higher compared to the presence of SDS (FIG. 12c). However, the fluorescence of RhB detected in the release media was extremely low, making it difficult to establish a reliable correlation between the rate of polymer degradation and the amount of dye released. In contrast, co-present SDS significantly increased the amount of dye detected in the media but restricted the degradation of polymer. Finally, post-added SDS resulted in the highest detection of degradation monomers and a 6-fold higher detection of RhB compared to the release without SDS. These observations suggest that the post-added surfactant approach is a feasible method to retrieve RhB from the degraded MPs while allowing the enzyme to degrade the polymer without any unfavorable interference of SDS.

Example 9. Fabrication-Induced Changes in Polymer Crystallinity and Their Impact on Enzymatic Degradation

DSC was performed by following the protocol in Example 2.

Degradation experiments

1mg of each PET formulation was suspended in 250 μ L of reaction mixture containing the FAST enzyme at a concentration of 10 μ M and incubated in a thermomixer at 1000 rpm and 50 °C. At 0.5, 1, 6, and 24 hours, 0.25% SDS was added, followed by further incubation in the thermomixer for an additional 1.5 hours. After centrifugation, the clear supernatant from each sample was then collected and analyzed using HPLC to quantify the PET degradation products.

Results and discussion

Although the protocol for release and degradation study has been optimized to be conducive for the enzymatic degradation of microparticles, we found that all the fabricated MPs degraded markedly lower compared to the LcPF-Pd, with a degradation rate of less than ~0.6% in 24 hours. In contrast, LcPF-Pd was found to degrade by ~6.6 % in the same duration (FIG. 13a). This could be due to physiochemical changes in the polymer as a result of the fabrication process and ingredients used. To further explore this, we fabricated microparticles under different conditions, including varying the % crystallinity of the polymer used (LcPF-p vs HcPF-p), incorporating dye (LcPF-RM vs LcPF-M and HcPF-RM vs HcPF-M), and using different fabrication techniques (HcPF-RM vs HcPF-RP).

DSC results (Table 5) showed that the % crystallinity of the polymer increased as the pristine polymer films (LcPF-p) were processed into particles by mechanical grinding (LcPF-Pd: 10.2% crystallinity), emulsion solvent evaporation technique (LcPF-M: 35% crystallinity), or film casting (HcPF-RP: 33.5% crystallinity). Interestingly, both the polymers, despite significant differences in their initial % crystallinity (LcPF-p: 2%, HcPF-p: 28%), eventually yielded microparticles of similar higher crystallinity (35%). The T_g values for the LcPF-p (68.1 ± 1.4 °C) increased upon mechanically grinding into powder (LcPF-PD: 78.9 ± 0.0 °C) but was not detected in all other microparticles prepared by film casting or emulsion technique, both of which involves solubilizing the PET in HFIP. Taken together, it can be inferred that when the polymer is solubilized in the HFIP and slowly reprecipitated in the ambient temperature, upon gradual evaporation of HFIP, the polymer chains are reorganized to form more ordered and shielded configuration resulting in increased crystallinity, causing remarkable reduction in the enzymatic degradation of the MPs.

Table 5. DSC parameters of various PET samples. T_g – Glass transition temperature, T_{cc} – cold crystallization temperature, ΔH_{cc} – enthalpy of cold crystallization, T_f – fusion temperature, ΔH_f – enthalpy of fusion.

Samples	T _g (°C)	T _{cc} (°C)	ΔH _{cc} (J g ⁻¹)	T _f (°C)	ΔH _f (J g ⁻¹)	Crystallinity (%)
LcPF-p	68.1±1.4	127.4±0.0	22.9±0.1	248±0.0	25.7±0.2	2.0±0.2
LcPF-Pd	78.9±0.0	132.5±0.9	17.2±0.2	246.3±0.0	31.5±0.8	10.2±0.4
LcPF-RM	-	-	-	246.3±0.2	44.9±0.3	32.0±0.2
LcPF-M	-	-	-	232.1±0.0	49.7±0.0	35.5±0.0
HcPF-p	-	-	-	253.0±1.4	40.1±2.7	28.6±1.9
HcPF-RM	-	-	-	251.3±0.1	49.2±0.0	35.1±0.0
HcPF-M	-	-	-	252.2±0.0	48.2±0.0	34.4±0.0
HcPF-RPd	-	-	-	250.1±0.0	47.4±0.0	33.4±0.0

Denotations:

LcPF and HcPF represents commercial low crystallinity PET film and high crystallinity PET film respectively.

-p indicates pristine form and -Pd indicates powder obtained by cryogenic grinding of the pristine PET films.

-RM and -M indicates RhB incorporated microparticles and blank microparticles of PET respectively, fabricated using emulsion technique.

-RPd indicates RhB incorporated powder obtained by film casting and subsequent cryogenic grinding.

FIG. 13b shows the SEM image of the samples. Microparticles produced via the emulsion solvent evaporation technique display a uniform, spherical, and partially-closed surface morphology. Conversely, film casting-grinding yields randomly shaped particles. While the latter exhibits slightly higher polymer degradation at each time point compared to the emulsion-based batches, the degradation levels remain notably low in comparison to LcPF-Pd. As such low degradation, this did not elicit a consistently meaningful release of RhB into the media for enzyme screening purposes.

Example 10. Enzymatic activity screening with two variant model enzymes of varying enzymatic activity using RhB-PET microparticles

Enzymatic activity screening

1 mg of dye-loaded microparticles was suspended in 250 μL of reaction mixture under two conditions: (1) containing the wild-type (WT) variant of PETase and (2) containing the FAST variant of PETase, each at a final concentration of 10 μM. The reaction mixtures were incubated in a thermomixer at 1000 rpm, with temperatures set to 30 °C for WT and 50 °C for

the FAST variant. At 0.5, 6, and 24 hours, 0.25% SDS was added to the mixtures, followed by incubation for an additional 1.5 hours. After centrifugation, the supernatant was then collected to measure the fluorescence of released Rhodamine B (RhB) and the absorbance of PET degradation products using HPLC.

Results and discussion

To improve the effective utilization of RhB-incorporated PET microparticles as a screening platform for enzymatic activity, it is crucial to ensure that the particles undergo significant degradation. This is essential to achieve a significant amount of dye release that is well-correlated with the rate of polymer degradation. To test this hypothesis, we synthesized linear PET, which exhibited a 7% degradation rate in 24 hours, even after being transformed into RhB-MPs using the emulsion-based technique described in Example 5, keeping the process parameters same. This rate is several times higher than that of microparticles synthesized using commercial PET above. The particles were subjected to degradation by two different variants of PETase enzyme, referred to as WT (wild type; natural) and FAST (genetically engineered), reportedly having different levels of enzymatic activity. FAST is known to have significantly higher enzymatic activity, and thus is able to degrade the polymer faster. This agrees with our results.

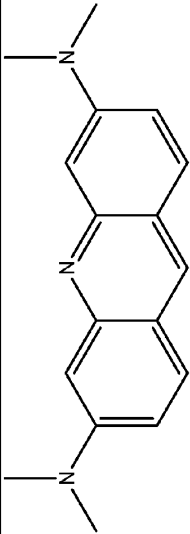
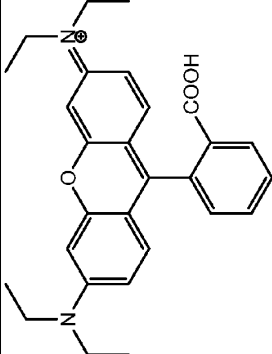
The RhB-MPs elicited the release of RhB at different rates (FIG. 14). The amount of dye released in the buffer (without any PETase) was the least of all across the time points. There was 30% more release of RhB in presence of WT enzyme, but the amount did not increase further over the study duration. In contrast, the particles released the highest amount of Rh-B in the presence of FAST enzyme. Also, there was a steep increase in the dye amount over the study duration; 140 units in the first 30 minutes which reached 220 units in 24 hours.

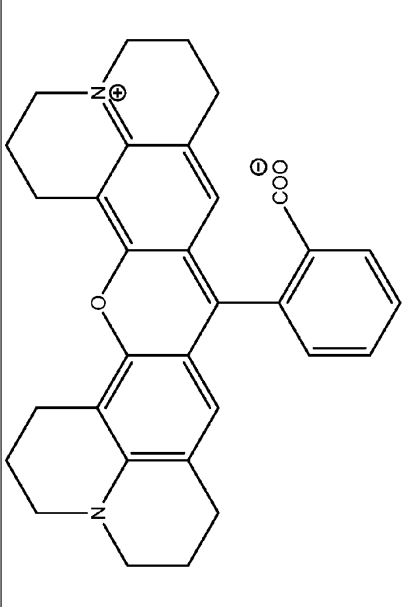
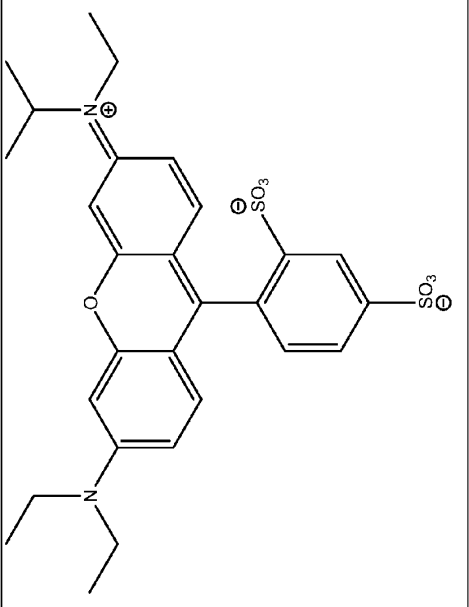
Upon analyzing the rate of polymer degradation, it was observed that the polymer remained relatively stable in buffer over the duration of the study. In the presence of WT enzyme, there was a modest degradation of less than 1% within 30 minutes, which increased to 2% within the next 24 hours. However, when exposed to the FAST enzyme, the polymer underwent a rapid degradation, with a 3% degradation observed within the first 30 minutes, and a gradual increase over time, resulting in a total of 7% degradation by the 24-hour mark. The release of dye from the microparticles thus showed a positive correlation with the enzymatic activity of the two enzymes tested, indicating that the platform was effective in detecting differences in the rates of polymer degradation.

The present disclosure thus demonstrated the potential of RhB-loaded PET microparticles as a valuable screening platform for enzymatic activity of PETase. However, it is important to acknowledge the limitations of this technique, particularly the inherent challenges posed by the resilience of the polymer to degradation upon fabrication into microparticles. This may limit the applicability of the technique for the screening of naturally occurring PET waste, which is likely to be even more resistant to degradation. Moving forward, it will be important to explore new methods for fabricating the dye incorporated microparticles that preserve the physicochemical attributes of the polymer. Future studies can also explore the use of different dyes or fluorescent molecules to enhance the sensitivity of the platform and extend its applicability to a wider range of enzymes. Such efforts could pave the way for broader applications of this promising screening technique in the field of enzymatic activity analysis.

Conclusion

Table 6. Encapsulation of dye in microparticles. FIG. 15 depicts the microstructures of PET microspheres after encapsulation.

Dyes	Structure	Dye charge	Encapsulation in PET microsphere	Correlation between Fluorescence and PET degradation
Acridine orange (AO)		neutral	Yes (Loading efficiency = 65%)	Good
Rhodamine B		cationic	Yes	Acceptable

Rhodamine 101		zwitterionic	Yes	Not good
Sulforhodamine		anionic	No	-

Claims

1. A polymeric microparticle comprising:
a polymeric material that is susceptible to enzymatic degradation; and
a dye encapsulated within the polymeric material.
2. The polymeric microparticle according to Claim 1, wherein at least a portion of the dye is released from the microparticle in an environment containing an enzyme suitable for degrading the polymeric material.
3. The polymeric microparticle according to Claim 1 or Claim 2, a polypropylene (PP), a polybutylene adipate terephthalate (PBAT), a polyamide (PA), and more particularly, a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), a polyethylene (PE), a polyurethane (PU) and a polystyrene (PS), copolymers thereof and blends thereof.
4. The polymeric microparticle according to Claim 3, wherein the polymeric material is selected from the group consisting of a polyamide (PA), a polystyrene (PS), a polyester-polyether-polyurethane-copolymer (PU/PCL copolymer); and a polyethylene terephthalate and polybutylene terephthalate blend (PET-PBT blend), and, more particularly, a polystyrene (PS), a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), and a polyethylene (PE).
5. The polymeric microparticle according to Claim 4, wherein the dye is a fluorescent dye, optionally wherein the dye is selected from one or more of the group consisting of methylene blue, toluidine blue O, acid fuchsin, alcian blue, sulforhodamine, and more particularly rhodamine B, and acridine orange.
6. The polymeric microparticle according to any one of the preceding claims, wherein the dye is selected from one more of the group consisting of methylene blue, toluidine blue O, acid fuchsin, and more particularly, acridine orange.
7. The polymeric microparticle according to Claim 6, wherein the dye is acridine orange.
8. The polymeric microparticle according to any one of the preceding claims, wherein the dye is present in an amount of from 0.5 to 3 wt%, such as from 0.6 to 2 wt%, such as from 0.61 to 1.93 wt%.

9. The polymeric microparticle according to any one of the preceding claims, wherein the microparticle has a crystallinity value (X_c) of from 20 to 40%, such as from 25 to 35%, such as from 27.5 to 32.4%.

10. The polymeric microparticle according to any one of the preceding claims, wherein the microparticle has a size of from 10 to less than 100 μm , such as from 30 to 90 μm , such as from 50 to 70 μm .

11. The polymeric microparticle according to any one of the preceding claims, wherein:

- (a) the polymeric material is a PET and the dye is acridine orange;
- (b) the polymeric material is a PBT and the dye is acridine orange;
- (c) the polymeric material is a PEN and the dye is acridine orange;
- (d) the polymeric material is a PS and the dye is acridine orange;
- (e) the polymeric material is a PE and the dye is acridine orange;
- (f) the polymeric material is a PA and the dye is acridine orange;
- (g) the polymeric material is a PU/PCL blend and the dye is acridine orange; or
- (h) the polymeric material is a PET-PBT blend and the dye is acridine orange,
- (i) the polymeric material is a PET and the dye is methylene blue;
- (j) the polymeric material is a PBT and the dye is methylene blue;
- (k) the polymeric material is a PEN and the dye is methylene blue;
- (l) the polymeric material is a PS and the dye is methylene blue;
- (m) the polymeric material is a PE and the dye is methylene blue;
- (n) the polymeric material is a PA and the dye is methylene blue;
- (o) the polymeric material is a PU/PCL blend and the dye is methylene blue;
- (p) the polymeric material is a PET-PBT blend and the dye is methylene blue;
- (q) the polymeric material is a PET and the dye is toluidine blue O;
- (r) the polymeric material is a PBT and the dye is toluidine blue O;
- (s) the polymeric material is a PEN and the dye is toluidine blue O;
- (t) the polymeric material is a PS and the dye is toluidine blue O;
- (u) the polymeric material is a PE and the dye is toluidine blue O;
- (v) the polymeric material is a PA and the dye is toluidine blue O;
- (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
- (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
- (y) the polymeric material is a PET and the dye is toluidine blue O;
- (z) the polymeric material is a PBT and the dye is toluidine blue O;
- (s) the polymeric material is a PEN and the dye is toluidine blue O;

- (t) the polymeric material is a PS and the dye is toluidine blue O;
- (u) the polymeric material is a PE and the dye is toluidine blue O;
- (v) the polymeric material is a PA and the dye is toluidine blue O;
- (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
- (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;

optionally wherein the polymeric material is selected from the list:

- (i) the polymeric material is a PET and the dye is acridine orange;
- (ii) the polymeric material is a PBT and the dye is acridine orange; or
- (iii) the polymeric material is a PEN and the dye is acridine orange.

12. The polymeric microparticle according to any one of the preceding claims, wherein the polymeric material is a PET and the dye is acridine orange.

13. A microfluidic device suitable for high throughput identification of one or more of an isolated enzyme, a cell lysate, and a microbe are suitable for degrading a polymeric material, the microfluidic device comprising a plurality of polymeric microparticles as described in any one of Claims 1 to 12.

14. A method of determining whether one or more of an isolated enzyme, a cell lysate, and a microbe are suitable for degrading a polymeric material, the method comprising the steps of:

- (aa) providing a plurality of polymeric microparticles according to Claims 1 to 12; and
- (ab) contacting the plurality of polymeric microparticles with the one or more of the isolated enzyme, the cell lysate, or the microbe in a suitable medium for a period of time and measuring the release of the dye over the period of time to determine whether the one or more of the isolated enzyme, the cell lysate, and the microbe are suitable for degrading the polymeric material in the polymeric microparticles.

15. The method according to Claim 14, wherein the method further comprises providing a control comprising a plurality of polymeric microparticles according to Claims 1 to 12 and a medium so as to provide a background reading over the period of time.

16. The method according to Claim 14 or Claim 15, wherein the dye release is measured using fluorescence.

17. A method of determining whether one or more of an isolated enzyme, a cell lysate, and a microbe are suitable for degrading a polymeric material, the method comprising the steps of:

- (i) providing a microfluidic device according to Claim 13; and
- (ii) contacting the plurality of polymeric microparticles in the microfluidic device with the one or more of the isolated enzyme, the cell lysate, and the microbe in a suitable medium for a period of time and measuring the release of the dye over the period of time to determine whether the one or more of the isolated enzyme, the cell lysate, and the microbe are suitable for degrading the polymeric material in the polymeric microparticles.

18. A method of making a plurality of polymeric microparticles according to any one of Claims 1 to 12, the method comprising the steps of:

- (ai) providing a first mixture comprising a polymeric material that is susceptible to enzymatic degradation, a dye and a first solvent, and second mixture comprising a surfactant and an anti-solvent for the polymeric material; and
- (aii) adding the first mixture to an agitated second mixture to provide the plurality of polymeric microparticles, wherein
the dye is soluble in the first solvent and the anti-solvent for the polymeric material.

19. The method according to Claim 18, wherein the first solvent is selected from one or more of the group consisting of toluene, dimethyl formamide, xylene, dichloroacetic acid, and, more particularly chloroform, and hexafluoropropan-2-ol, optionally wherein the first solvent is a mixture of chloroform and hexafluoropropan-2-ol, further optionally wherein the volume to volume ratio of chloroform to hexafluoropropan-2-ol of from 1:1 to 10:1, such as from 2:1 to 5:1, such as about 4:1.

20. The method according to Claim 18 or Claim 19, wherein one or more of the following apply:

- (bi) the anti-solvent is water (e.g. ultra-pure water);
- (bii) the surfactant is a polyvinyl alcohol or poly(vinyl alcohol-co-vinyl acetate) (e.g. a polyvinyl alcohol or poly(vinyl alcohol-co-vinyl acetate) (e.g. 88% vinyl alcohol and 12% vinyl acetate repeating units) with a M_w of about 67,000);
- (biii) the surfactant is present in an amount of from 1 mg surfactant per gram of anti-solvent to 100 mg surfactant per gram of anti-solvent, such as from 10 mg surfactant per gram of anti-solvent to 50 mg surfactant per gram of anti-solvent, such as from 20 mg surfactant per gram of anti-solvent to 30 mg surfactant per gram of anti-solvent.

21. The method according to any one of Claims 18 to 20, wherein the polymeric material is selected from the group consisting of a polypropylene (PP), a polybutylene adipate terephthalate (PBAT), a polyamide (PA), and more particularly, a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), a polyethylene (PE), a polyurethane (PU) and a polystyrene (PS), copolymers thereof and blends thereof,

optionally wherein the polymeric material is selected from the group consisting of a polyamide (PA), a polystyrene (PS), a polyester-polyether-polyurethane-copolymer (PU/PCL copolymer); and a polyethylene terephthalate and polybutylene terephthalate blend (PET-PBT blend), and, more particularly, a polystyrene (PS), a polyethylene terephthalate (PET), a polybutylene terephthalate (PBT), a polyethylene naphthalate (PEN), and a polyethylene (PE),

yet more optionally wherein the the polymeric material is a PET, a PBT, a PEN, a copolymer thereof, and a blend thereof, optionally wherein the polymeric material is a PET.

22. The method according to any one of Claims 18 to 21, wherein the dye is a fluorescent dye, optionally wherein the dye is selected from one or more of the group consisting of methylene blue, toluidine blue O, acid fuchsin, alcian blue, sulforhodamine, and more particularly rhodamine B, and acridine orange.

23. The method according to any one of Claims 18 to 22, wherein the dye is present in the first mixture in a concentration of from 0.001 to 10 mg/mL, such as from 0.005 to 1 mg/mL, such as from 0.01 to 0.05 mg/mL, such as about 0.299 mg/mL

24. The method according to any one of Claims 18 to 23, wherein:

- (a) the polymeric material is a PET and the dye is acridine orange;
- (b) the polymeric material is a PBT and the dye is acridine orange;
- (c) the polymeric material is a PEN and the dye is acridine orange;
- (d) the polymeric material is a PS and the dye is acridine orange;
- (e) the polymeric material is a PE and the dye is acridine orange;
- (f) the polymeric material is a PA and the dye is acridine orange;
- (g) the polymeric material is a PU/PCL blend and the dye is acridine orange; or
- (h) the polymeric material is a PET-PBT blend and the dye is acridine orange,
- (i) the polymeric material is a PET and the dye is methylene blue;
- (j) the polymeric material is a PBT and the dye is methylene blue;
- (k) the polymeric material is a PEN and the dye is methylene blue;
- (l) the polymeric material is a PS and the dye is methylene blue;
- (m) the polymeric material is a PE and the dye is methylene blue;

- (n) the polymeric material is a PA and the dye is methylene blue;
 - (o) the polymeric material is a PU/PCL blend and the dye is methylene blue;
 - (p) the polymeric material is a PET-PBT blend and the dye is methylene blue;
 - (q) the polymeric material is a PET and the dye is toluidine blue O;
 - (r) the polymeric material is a PBT and the dye is toluidine blue O;
 - (s) the polymeric material is a PEN and the dye is toluidine blue O;
 - (t) the polymeric material is a PS and the dye is toluidine blue O;
 - (u) the polymeric material is a PE and the dye is toluidine blue O;
 - (v) the polymeric material is a PA and the dye is toluidine blue O;
 - (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
 - (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
 - (y) the polymeric material is a PET and the dye is toluidine blue O;
 - (z) the polymeric material is a PBT and the dye is toluidine blue O;
 - (s) the polymeric material is a PEN and the dye is toluidine blue O;
 - (t) the polymeric material is a PS and the dye is toluidine blue O;
 - (u) the polymeric material is a PE and the dye is toluidine blue O;
 - (v) the polymeric material is a PA and the dye is toluidine blue O;
 - (w) the polymeric material is a PU/PCL blend and the dye is toluidine blue O;
 - (x) the polymeric material is a PET-PBT blend and the dye is toluidine blue O;
- optionally wherein the polymeric material is selected from the list:
- (i) the polymeric material is a PET and the dye is acridine orange;
 - (ii) the polymeric material is a PBT and the dye is acridine orange; or
 - (iii) the polymeric material is a PEN and the dye is acridine orange.

25. The method according to Claim 24, wherein the polymeric material is a PET and the dye is acridine orange.