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Ion implantation treatment of beads for covalent binding of molecules: Application to bioethanol production using thermophilic beta-glucosidase

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ABSTRACT

We have achieved plasma immersion ion implantation (PIII) treatment of beads and powders using a specially designed plasma treatment system. This simple one-step production of functionalized beads provides an attractive alternative to current commercial functional beads, for which proteins must be chemically attached using linkers.

Using the enzyme beta-glucosidase as an example we show that PIII treatment of polyethylene beads enables covalent binding with increased activity of the enzyme compared to the untreated beads. Covalent binding was confirmed using detergent washing. The covalently immobilized enzyme has a broader pH range over which it has high activity than the enzyme in solution. The stability of the immobilized molecules was examined using reaction rate as a function of temperature and was shown to be significantly higher on the PIII treated beads compared to untreated beads. We attribute the increased enzyme activity on PIII treated beads to increased protein binding density and better retention of conformation. The results of this work are of significance in the production of ethanol using a flow process. Covalent binding to beads allows more robust attachment for high flow rates, high activity, large surface area and a broad operating pH range. Treatment could be easily adapted for a range of applications such as linking drugs, dyes and proteins to particles of an appropriate size.

1. Introduction

Immobilizing biomolecules such as enzymes on particles allows a greater area of functionalized surface to be conveniently used in processing. A large surface area is especially useful where immobilized proteins are to be used in a flow process. Such a flow process may provide significant efficiency improvements in enzymatic processing in the food, chemical and biofuel industries. If the flow rates are high, it is important that the enzymes are strongly attached, preferably covalently.

Plasma immersion ion implantation (PIII) treatment of plastics has been shown to be useful for the covalent binding of proteins to plastic surfaces without the use of linkers [1,2]. The amount of protein attached per unit area is also higher for PIII treated surfaces than it is for untreated surfaces [3]. A quantitative theory for the covalent binding behaviour of PIII treated surfaces has been proposed [1]. In this theory, the ion interactions with the polymer produce a reservoir of radicals in the subsurface. The radicals diffuse to the surface where they form covalent bonds to physically adsorbed species. In principle, the PIII treatment enables the treated surface to bind almost any molecule. This means many small molecules as well as large biomolecules can be attached covalently to a plastic surface without the need for linkers. The process appears to operate well on many polymers, including polyethylene, polystyrene, nylon, PTFE, PEEK, polypyrrole, polycarbonate and PET.

Linker free covalent immobilization using plasma immersion ion implantation treatments have so far only been studied on flat surfaces. There are some examples of plasma treatment of powders without the energetic ion bombardment that PIII treatment provides. Plasma treatment has been shown to improve the wettability and flowability of fine powders [4] and to increase the stability of acrylic acid on polystyrene beads [5]. The wettability of polyethylene powders has been significantly improved by treatment in an oxygen plasma discharge at 133 Pa [6].

Previous work shows that protein immobilization onto ammonia plasma treated polypropylene beads can be achieved using linker molecules [7,8]. Plasma functionalization of polystyrene and polyvinylchloride microspheres with allylamine or acrylic acid resulted in higher protein retention on plasma treated beads [9].

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Potential uses for PIII treated plastic particles include as drug delivery platforms, in affinity columns for protein purification and as protein and dye functionalized beads for use in cell assays and flow processes. The fact that many molecules will bind covalently to PIII treated polymers allows the functionalized beads to be used in the presence of detergents as are used in many assays and that remove non-covalently bound molecules from the surface of polymers. In processes where there is a strong liquid flow velocity, the stronger binding created by covalent bonds facilitates retention of the immobilized molecules, preventing them from being washed out of the reaction vessel. Currently commercially available functional beads require chemical processing to provide linker molecules with specific groups at the ends that react chemically with groups on the bead and protein surfaces [10]. The disadvantages of this approach are multiple stage wet chemistry, side reactions, variable yields and solvent waste. A recent new approach overcomes these issues by expressing protein functionalized polyester beads directly from engineered bacteria [11]. In this paper, we describe a dry processing technique which also overcomes the disadvantages of chemical-linker methods and which can be used on most carbon based polymers.

The aim of this work is to demonstrate first that a PIII treatment can be successfully carried out on granules and particles and second that protein can be strongly immobilized on the surface. Polyethylene granules are used as a test material. The polyethylene granules float in aqueous solution but are dense enough for them to be completely covered by the solution so the attached protein does not dehydrate. The example proteins we used for attachment studies were beta-glucosidase, one of the enzymes required in ethanol production from cellulosic waste and horseradish peroxidise (HRP), a readily available enzyme with a convenient colorimetric assay for activity.

2. Materials and methods

The UHMW polyethylene granules (2 mm) were from Goodfellow (Cat. No. ET306300). They are not perfectly spherical but are described as having an "approximately regular shape". UHMW polyethylene powder (150 μm) was from Goodfellow (ET306010). The plasma chamber used to treat the granules has been described elsewhere [12]. We used Nitrogen at a working pressure of 4.3×10^{-3} Torr as the gas for generating the plasma. An RF powered helicon inductively coupled antenna was used to sustain the discharge. RF forward and reflected powers were 100 W and zero, respectively. For PIII treatment, the granules were placed in a shaker apparatus that consists of a cylindrical stainless steel chamber (18 mm (d) × 25 mm (w)) surrounded by a metallic mesh, allowing the particles to be biased with respect to the plasma while being shaken to present all of the surfaces to the plasma. The chamber was placed into a teflon holder on a mechanical shaker. 5 kV pulses of length 20 μs were applied to the stainless steel chamber containing the polyethylene granules at a repetition rate of 50 Hz while it was subjected to continuous mechanical shaking. Granules were treated for 6 min with constant shaking for all experiments unless otherwise stated.

2.1. Production and purification of beta-glucosidase

Beta-glucosidase from *Caldicellulosiruptor saccharolyticus* was cloned and purified as previously described [13]. Protein expression was induced by two methods: (a) IPTG (isopropyl β-D-1-thiogalactopyranoside) induction and (b) lactose induction following the auto-induction method of Studier [14]. Protein was also grown without induction for comparison. Media used for induction with and without IPTG was LB media (GE Healthcare US75852). The media used for auto-induction was ZYM-5052 taken from Studiers auto-induction paper.

2.2. FTIR spectra

ATR-FTIR spectra were recorded on PIII-treated and untreated polyethylene particles and sheets using Digilab FTS 7000 FTIR spectrometer with a Harrick ATR accessory. A germanium crystal and 45° angle of incidence were used and 5000 scans were taken at a resolution of 4 cm^{-1}.

2.3. Protein attachment

Beta-glucosidase was attached to PIII treated and untreated granules by incubating the granules overnight in beta-glucosidase (100 μg/ml) in 10 mM phosphate buffer pH 7 in a 50 ml Falcon tube. Granules were washed once in 10 mM PO_4 and then transferred to a clean Falcon tube and washed a further 5 times. Solution was removed by pipetting. For the experiment using horseradish peroxidase (HRP, Sigma P6782) the attachment protein concentration and washing steps were the same. For Triton X-100 (Sigma T8787) experiments, after protein attachment and washing as previously described samples were then incubated in 1% detergent in 10 mM phosphate buffer pH 7 for 60 min at room temperature then washed twice with 10 mM phosphate buffer pH 7 to remove the Triton X-100.

Table 1
Comparison of beta-glucosidase yields using different protein induction methods.

	LB media	LB media + IPTG	Auto-induction media
A 600 nm	2.8	1.5	6
Protein yield/litre (mg)	30	20	180–250

2.4. Protein assay

Protein activity was assayed by adding 5 granules to 0.5 ml of 10 mM 4-Nitrophenyl β-D-glucopyranoside (Sigma N7006) or 10 mM 2-Nitrophenyl β-D-glucopyranoside (Sigma N8016), which are artificial substrates for glucosidase, in 0.05 M acetate buffer pH 5 in an eppendorf tube and incubated for 1 h at 37 °C. For pH experiments 0.05 M citrate was used for pH 2–3.5, 0.05 M acetate buffer was used from pH 4 to 6 and 50 mM phosphate buffer was used at pH 6.5 and 7. Aliquots were taken and diluted in an appropriate volume of unreacted 10 mM 4-Nitrophenyl β-D-glucopyranoside bringing the volume to 100 μl. Sodium carbonate was added (100 μl) and the colour measured at 405 nm. HRP coated granules were assayed by incubating 5 granules in 0.5 ml TMB (Sigma T0440) at room temperature.

For time course experiments, after protein attachment overnight and washing, granules were assayed for enzyme activity and this was called day 0. Granules were left in washing buffer which was changed every 3 days with fresh washing buffer and granules were assayed for remaining enzyme activity for up to 2 weeks.

The data to produce Arrhenius plots was obtained by measuring the reaction rate as a function of temperature in 0.05 M acetate buffer at pH 5. Five 10 μl aliquots were taken at various times for each point from the 0.5 ml solution.

3. Results and discussion

3.1. Expression of recombinant beta-glucosidase

Table 1 shows the expression levels of protein as a function of various methods of induction. Protein expression was observed without any inducer, implying the media contained some lactose. Recombinant protein expression with or without IPTG induction gave a similar protein yield. However induction with lactose using Studier's auto-induction method resulted in a large increase in protein expression. Table 1 shows that it is possible to obtain 10 times the amount of protein with Studiers auto-induction method with only 4 times the final cell density compared to IPTG induction.

Lactose is a substrate for our beta-glucosidase (unpublished data) and perhaps the presence of the substrate in the auto-induction media encourages further protein expression to give the high levels of expression we observe. Since this is a potentially a useful enzyme for commercial applications, high levels of protein expression are desirable. It is quite straightforward to produce grams of beta-glucosidase even whilst using shaking flasks instead of a purpose built fermenter. Large amounts of glucosidase from *Caldicellulosiruptor bescii* have also been reported using auto-induction in *E. coli* [15].

3.2. FTIR spectra

Due to poor contact with the ATR crystal it was not possible to get FTIR spectra of the treated granules. Fig. 1 shows FTIR spectra of a flat sheet of UHMWPE (1 cm × 1 cm) which had been PIII treated for 6 min in the same shaker used to PIII treat the granules, but without shaking during the treatment. Also shown is the spectrum for 150 μm PE powder (50 mg) which had been treated