

**Characterization and application of a sterol esterase
immobilized on polyacrylate epoxy-activated carriers
(Dilbeads™)**

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ABSTRACT

The sterol esterase from the ascomycete *Ophiostoma piceae* was immobilized on novel polyacrylate-based epoxy-activated carriers (Dilbeads™). Six supports with particle sizes between 120-165 μm were prepared varying the composition of monomers, crosslinkers and porogens. Their surface areas and porosities were determined by N₂ adsorption and mercury intrusion porosimetry. The pore volumes ranged from 0.63 to 1.32 cm³/g, but only Dilbeads™ RS and NK had narrow pore size distributions (with maxima at 33.5 and 67.0 nm, respectively). The distribution of the enzyme in the support was studied by fluorescence confocal microscopy. The immobilized esterase on Dilbeads™ TA showed a significant pH and thermal stability and was assayed in the continuous hydrolysis of cholesteryl esters -present in the pulp industry process waters-.

Key words: Covalent immobilization, Epoxy carriers, Cholesterol esterase, Confocal microscopy, Pitch control.

1. INTRODUCTION

For the industrial development of biocatalytic processes an effective immobilization method is commonly required to allow the reuse of enzymes or continuous processing [1,2] Different strategies have been proposed to immobilize enzymes, based on adsorption [3,4], covalent binding [5], granulation [6], entrapment in polymers [7] and cross-linking of enzyme crystals or protein aggregates [8].

Covalent immobilization has the advantage of forming strong and stable linkages between the enzyme and the carrier that result in robust biocatalysts [5]. Covalent attachment also eliminates the loss of activity caused by enzyme leakage from the support. Different materials, e.g. crosslinked dextrans (Sephacrose), polysaccharides (agarose) or porous silica, can be chemically activated by different approaches to covalently attach enzymes [9]. However, the number of commercialized activated carriers for covalent immobilization is relatively small compared with available enzyme adsorbent materials.

Epoxy(oxirane)-activated materials, such as Eupergit C [10,11] or Sepabeads [12,13] are very attractive because of their high reactive groups density and the simple chemistry for covalent attachment of the enzyme to the support. In this work we describe for the first time a new class of epoxy-activated supports (Dilbeads™), based on the combination of three or four types of acrylic monomers with varying porogen concentration, prepared with a range of pore sizes to cover the spatial requirements of different enzymes. These materials are rigid and do not swell in water as occurs with the polyacrylamide-based Eupergit C. Dilbeads™ are epoxy(oxirane)-activated, so they bind enzymes as a function of pH through different nucleophiles: at neutral or slightly alkaline pH with the thiol groups, at pH>8 with the amino groups and at pH>11 with phenolic groups of tyrosines [12]. The spatial

distribution of the enzyme in the carrier bead was studied by confocal laser scanning microscopy, which non-invasively gathers optical slices from a macroscopic bead.

To test the applicability of these carriers for enzyme immobilization, we have covalently attached to Dilbeads™ the sterol esterase from the ascomycete *Ophiostoma piceae*, which can be used for hydrolysis of cholesteryl esters during pulp manufacture [14]. These colloidal particles accumulate in pulp or machinery forming “pitch deposits” or remain suspended in the process waters. The application of enzymes for the biotreatment of wastewaters (enzymatic bioremediation) is rapidly expanding as it offers some advantages compared with other technologies [15].

2. EXPERIMENTAL SECTION

2.1. Materials

Cholesterol, cholesteryl oleate, *p*-nitrophenyl butyrate, polyoxyethylene 10 tridecyl ether (Genapol X-100), fluorescein isothiocyanate and hydranal-composite 5 were purchased from Sigma. Sterol esterase was produced as described elsewhere [16]. All other reagents and solvents were of the highest available purity and used as purchased.

2.2. Synthesis and characterization of Dilbeads™ carriers

All the carriers were synthesized in 500 ml flat-bottom cylindrical reactors, previously purged with nitrogen, fitted with a reflux condenser, a mechanical stirrer and a thermometer. Suspension polymerization was carried out to produce different types of polymer beads, by using a judicious mix of acrylate monomers, cross-linkers and porogens. After the polymerization, the beads were filtered and washed thoroughly. Dilbeads™ is a proprietary product of Fermenta Biotech Ltd., and the process of manufacture is detailed in an Indian patent application [17]. The polymer beads formed by the above proprietary process were further used in the immobilization experiments.

Mercury intrusion porosimetry analyses of the supports were performed using a Fisons Instruments Pascal 140/240 porosimeter. To ensure that the samples were moisture free, they were dried at 100°C overnight prior to measurement. The recommended values for the mercury contact angle (141°) and surface tension (484 mN/m) were used to evaluate the pressure/volume data by the Washburn equation,

assuming a cylindrical pore model [18]. The particle size distributions of the supports were determined by analysis of the intrusion curve, which in the case of a finely divided powder gives information on the interparticle porosity. From the total porosity of the material -assuming spherical particles- the packing factor and subsequently the particle size distribution were calculated according to the Mayer-Stowe theory [19]. The specific surface area (S_{BET}) of the supports was determined from analysis of nitrogen adsorption isotherms at -196°C , using a Micromeritics ASAP 2010 device. The samples were previously degassed at 100°C for 12 h to a residual vacuum of $5 \cdot 10^{-3}$ torr, to remove any loosely-held adsorbed species. Water content of the supports was assayed using a DL31 Karl-Fisher titrator (Mettler). Scanning electron microscopy (SEM) was performed using an XL3 microscope (Philips) on samples previously metallized with gold.

2.3. Preparation and characterization of immobilized sterol esterase

DilbeadsTM (1 g) were mixed with 5 ml of crude sterol esterase in 0.3 M potassium phosphate buffer (pH 8.0). The mixture was incubated for 72 h at 4°C with roller shaking. The biocatalyst was then filtered using a glass filter (Whatman), washed (3 x 10 ml) with 1 M potassium phosphate buffer (pH 8.0), dried under vacuum and stored at 4°C .

To characterize the distribution of sterol esterase in DilbeadsTM, we used fluorescence confocal microscopy with proteins previously labeled with fluorescein isothiocyanate (FITC). The protein to be labeled was first dissolved in 0.05 M carbonate buffer, pH 9.0, to a final concentration of approx. 4.5 mg/ml. FITC dissolved in dimethylformamide was then added to a FITC/protein ratio of 5 $\mu\text{g}/\text{mg}$. The reaction was then performed at room temperature for 1 h. The labeled protein

was purified from unbound FITC by gel chromatography using a pre-packed PD-10 column (Amersham Biosciences). Fluorescence confocal microscopy was performed with a Leica TCS SP2 confocal laser scanning microscope (CLSM) equipped with nine excitation lines and software for image processing. A 40.0 x 1.25 oil immersion objective was used for all measurements and the pinhole aperture was set to 1.50 Airy (122 μm). The laser provided excitation of FITC at 488 nm and emitted fluorescent light was detected at 520 nm.

The pH stability was measured at 25 °C incubating the enzyme in 20 mM citrate-phosphate-borate buffer at different pH values. In the case of immobilized enzyme, the amount of biocatalyst added was adjusted to approx 0.5 mg per ml. The remaining activity was measured using the standard activity assay with *p*-nitrophenyl butyrate (pNPB) after different times in reaction. The thermostability studies were performed in 20 mM citrate-phosphate-borate buffer (pH 6.0) incubating the enzyme (0.5 mg immobilized biocatalyst per ml) at 45 or 60 °C. The remaining activity was measured with pNPB at different times. The activity was assayed spectrophotometrically following *p*-nitrophenol release ($\epsilon_{410} = 15200 \text{ M}^{-1} \text{ cm}^{-1}$) from 1.5 mM pNPB in 20 mM citrate-phosphate-borate buffer (pH 7.2).

2.4. Continuous removal of cholesteryl esters in a packed-bed reactor.

The sterol esterase immobilized in DilbeadsTM TA (approx. 1 g) was packed in a HR 5/5 column (0.5 x 5 cm, 1 ml, Amersham Biosciences) connected to a peristaltic pump (model P-1, Amersham Biosciences). The feed solution contained 2 g/l cholesterol oleate, 10% (v/v) Genapol X-100, 0.1 M KCl and 1 mM Tris-HCl (pH 8.0). The homogeneity of the feed solution was ensured by magnetic stirring. This configuration did not present any recycle loop, and the solution was passed at 15

ml/h. After the passage of at least 10 column volumes (10 ml) of feed solution to equilibrate the system (time 0), samples were collected at the exit of the reactor, centrifuged 5 min at 6000 rpm using an eppendorf with a 0.45 μm Durapore[®] membrane (Millipore) and analyzed by HPLC. An isocratic pump (model 515, Waters) coupled to a Nucleosil C-18 column (4.6 x 150 mm) (Análisis Vínicos, Spain) was used. The mobile phase was acetonitrile:2-propanol 60:40 (v/v), conditioned with helium, at 1.0 ml/min. The column temperature was kept constant at 30°C. A UV-Vis detector (model 9040, Spectra-Physics) was used and set to 206 nm. The data obtained were analyzed using the Varian Star Chromatography Workstation 6.41.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of Dilbeads™ supports

Dilbeads™ are suitable for covalent immobilization of enzymes for industrial applications because of their high mechanical stability and non-swelling in aqueous or organic media. We prepared six different Dilbeads™ carriers (EZ, DVK, NK, RS, SZ and TA) varying the monomer, crosslinkers and porogen type and quantities. As the crosslinking density and the choice of the porogen greatly influence the pore distribution, different combinations were made to vary the pore size distribution.

Despite the presence of some small particles, the size was quite uniform, in the range 120-165 μm . The textural properties of the supports were measured by a combination of nitrogen adsorption and mercury porosimetry analyses. Table 1 summarizes the main textural parameters (BET surface area, particle size, mean pore diameter, etc.) and Fig. 1 shows the total pore volume of these carriers. Dilbeads™ TA presented the highest total pore volume (1.32 cm^3/g), and Dilbeads™ RS the lowest value (0.63 cm^3/g). The contribution of mesopores (20-500 \AA) to the total pore volume was higher than 50%, except for Dilbeads™ NK (22%) and DVK (47%). The pore distribution curve of Fig. 2 shows two groups of supports: the first is formed by Dilbeads™ RS and NK that had narrow pore size distributions with a well defined maximum (at 335 and 670 \AA , respectively, Fig. 2A), whereas the other materials showed a broader range of pore diameters with several maxima (Fig. 2B). The specific surface area varied from 67 m^2/g for Dilbeads™ DVK to 311 m^2/g for Dilbeads™ TA (the latter material also presented a notable contribution of micropores, 0-20 \AA). The morphology and porous structure of Dilbeads™ supports is illustrated in the SEM pictures of Dilbeads™ TA (Fig. 3). For all these synthesized supports, the average pore size values (in the range 200-1500 \AA) indicate that most

biomolecules may diffuse into the carrier, which should result in a higher volumetric activity of the biocatalyst [20].

3.2. Immobilization of sterol esterase in Dilbeads™.

The *O. piceae* sterol esterase hydrolyzes *p*-nitrophenyl esters, triglycerides and cholesterol esters [14]. The hydrolysis efficiency increases with the length of the fatty acid [16]. The immobilization of sterol esterases from other microorganisms has been previously studied for the determination of serum cholesterol in diagnostics [21], the synthesis of cholesteryl esters [22] or their application in biosensors [23].

The immobilization was carried out at high ionic strength because for this type of epoxy-activated carriers it has been postulated that, in a first step, a salt-induced association between the macromolecule and the support surface takes place, which increases the effective concentration of nucleophilic groups on the protein close to the epoxide reactive sites [24]. The immobilization was performed at pH 8.0, at which the amino (lysines) and thiol (cysteines) groups of the enzyme are able to bind to the support [25].

The activity recovery in Dilbeads™ TA (approx. 20%) was similar to that obtained with other epoxy-activated supports such as Eupergit C [26,27] and Sepabeads [12]. In this context, Cao has recently reported that retention of activity is usually low (below 40%) with oxirane-activated supports [28]. In general, we observed that the higher the pore size of the carrier the higher the recovery of activity. Thus, the recovered activity using Dilbeads™ RS and NK (with average pore sizes of 33.5 and 67.0 nm, respectively) was only 3% and 10%, respectively, which are substantially lower than the obtained with the more porous Dilbeads™ TA. The activity loss, which has been reported by numerous researchers, may be related to several factors, such as the orientation of active site towards the surface,

conformational changes caused by the covalent bonds formed, or mass transport limitations of substrate and/or products [28,29].

3.3. Characterization of the immobilized biocatalyst

To characterize the distribution of sterol esterase within Dilbeads™, we used fluorescence confocal microscopy, which renders spatial information about the distribution of fluorescent compounds over the radius of an immobilized bead [29]. This technique has been employed to visualize the distribution of biomolecules throughout the bead (esp. in hydrogels) as well as to evaluate restrictions to diffusion within the support [29-32]. A typical confocal image for FITC-labeled enzyme immobilized in Dilbeads™ TA is shown in Fig. 4, varying the observation depth. It is worth noting that the enzyme was not uniformly distributed in the beads. Most of the enzyme molecules are confined in a surface layer of approx. 10.5 μm width. This depth of the enzyme layer was very similar analyzing beads of different radius. Thus, there is an apparent restriction for diffusional transport into the interior of the bead, which can be caused, among other factors, (1) by the tortuosity of the pore structure, or (2) by the steric hindrance exerted by the enzyme molecules that are immobilized in the shell of the particle.

We also analyzed the thermostability of soluble and immobilized biocatalysts at 45 and 60 °C. Fig. 5 shows that the native enzyme lost 80% of its initial activity in 2.5 h at 60 °C, whereas the immobilized one maintained 50% activity after 24 h. At 45 °C, the immobilization also stabilized the sterol esterase although not so substantially. The pH-stability of the immobilized sterol esterase was also investigated (data not shown). The enzyme was incubated for 24 h at different pH values and the residual activity assayed with pNPB. Interestingly, a significant

stabilization effect was observed at pH 8.0, the optimum pH for this enzyme. Thus, the residual activity after 24 h was 31.5% and 97.5% for native and immobilized enzyme, respectively. Similar stabilization effects against pH and temperature have been described with other related supports [33-35].

3.4. Continuous removal of cholesteryl esters in a packed-bed reactor.

O. piceae sterol esterase possesses a high interest for pitch removal in paper pulp manufacturing since sterol esters are problematic compounds in the processing waters as they form deposits that contain high amounts of oleate and linoleate esters of sterols [36,37]. To mimic the treatment of processing waters by sterol esterase we prepared a continuous packed-bed reactor containing approx. 1 g of immobilized enzyme (in Dilbeads™ TA). The feed solution contained 2 g/l sterol oleate in the working buffer containing 10% (v/v) Genapol X-100 to emulsify the substrate (see Experimental Section).

The immobilized enzyme was used over five days in a packed-bed reactor fed with 15 ml/h substrate (residence time: 4 min). A loss of activity was observed during the first 24 h; after this time, the performance of the bioreactor was quite stable in terms of cholesteryl oleate hydrolysis, with a productivity of approx. 6 g h⁻¹ kg⁻¹ biocatalyst. This biphasic behaviour is typical of covalently-attached enzymes, and is commonly attributed to the heterogeneity of the immobilized biocatalyst (with enzyme molecules differing in the number of covalent bonds with the support and/or in the orientation of their active site) [25]. However, the monophasic curves obtained in the thermostability studies using pNPB (Fig. 5) suggest that the biphasic behavior in the fixed-bed reactor may be basically attributed to the drastic conditions of the assay, in

particular the presence of a high concentration (10% v/v) of a non-ionic surfactant (Genapol X-100).

CONCLUSIONS

Dilbeads™ are very promising carriers for enzyme immobilization. Their main advantages are their easily-modulated porosity, high mechanical stability and non-swelling in water. Immobilization of enzymes on these materials is rapid and easy both at laboratory and industrial scales. We have demonstrated that the enzyme is mostly confined in the outer 10 μm layer of the beads, probably due to diffusional restrictions during the process of contacting the protein and the carrier. The use of the above biocatalysts for the continuous hydrolysis of pitch deposits in the process waters of the pulp industry is only one example of their technological applications [38].

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Table 1. Properties of the Dibeads™ supports.

Support	Particle size (μm) ^a	S_{BET} (m^2/g) ^b	Pore volume (cm^3/g) ^c		Average pore size (nm) ^d	Water content (%) ^e
			Mesopores	Total		
Dilbeads EZ	133	84	0.54	0.97	31.5, 38.0, 51.5, 64.5	13
Dilbeads DVK	159	67	0.49	1.04	29.5, 49.5, 67.0, 95.5	8
Dilbeads NK	103	81	0.18	0.80	67.0	20
Dilbeads RS	164	84	0.50	0.63	33.5	9
Dilbeads SZ	124	103	0.62	0.73	27.5, 39.0	7
Dilbeads TA	138	311	0.99	1.32	27.5, 39.5, 68.5, 99.0	12

^a Determined by Hg porosimetry, considering a symmetric distribution of particle size.

^b Measured by N_2 adsorption.

^c By combination of N_2 isotherms and Hg porosimetry.

^d The maxima in the pore size distribution curve are indicated.

^e Determined by Karl-Fisher titration.

Figure Legends

Fig. 1. Total pore volume of Dilbeads™ supports determined by combination of N₂ adsorption and mercury intrusion porosimetry data.

Fig. 2. Pore size distribution of Dilbeads™ supports: (A) carriers with a narrow distribution of pore sizes; (B) carriers with a wide distribution of pore sizes.

Fig. 3. Scanning electron micrographs of Dilbeads™ TA and DVK: (A) 60x; (B) 500x; (C) 8000x; (D) 40000x.

Fig. 4. Confocal images of FITC-labeled crude cholesterol esterase immobilized on Dilbeads™ TA. The images were obtained by taking different deep z-section scans with 5 μm depth increment between each picture from A to D.

Fig. 5. Thermal stability at 45 °C (●) and 60 °C (○) of sterol esterase from *O. piceae*: (A) soluble; (B) immobilized on Dilbeads™ TA. Conditions described in Experimental Section.

Fig. 1

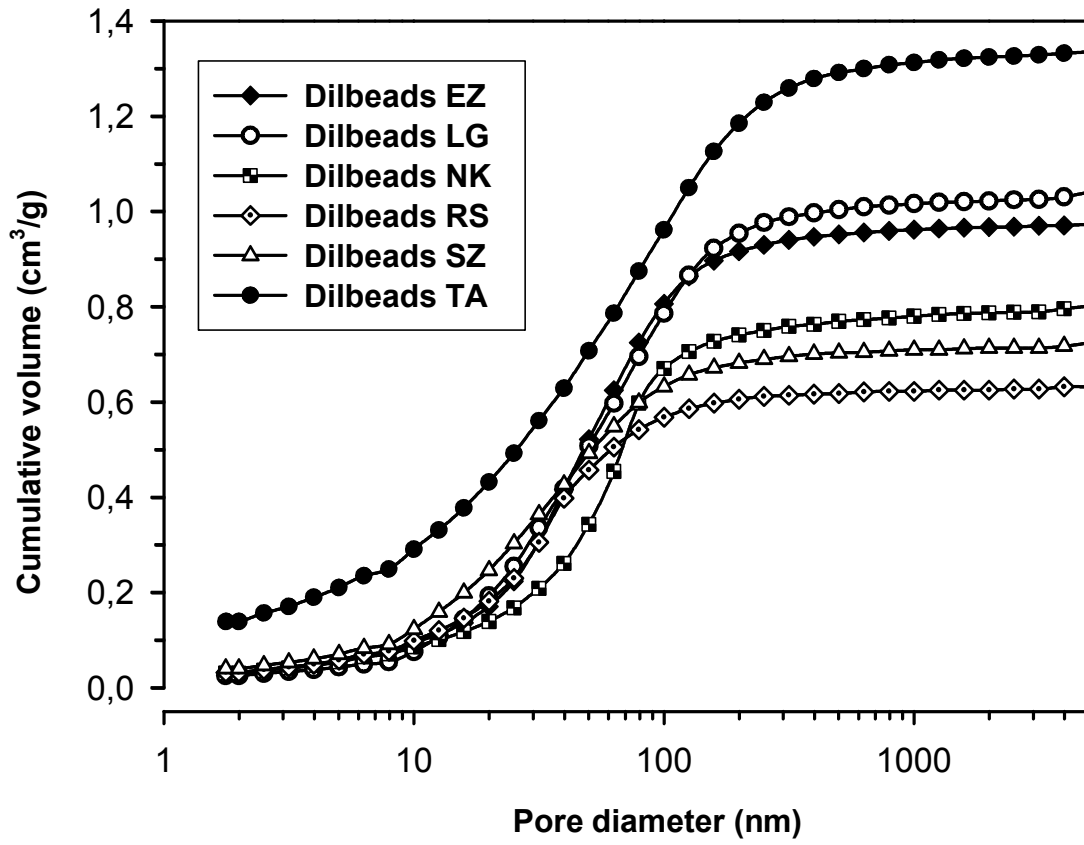


Fig. 2

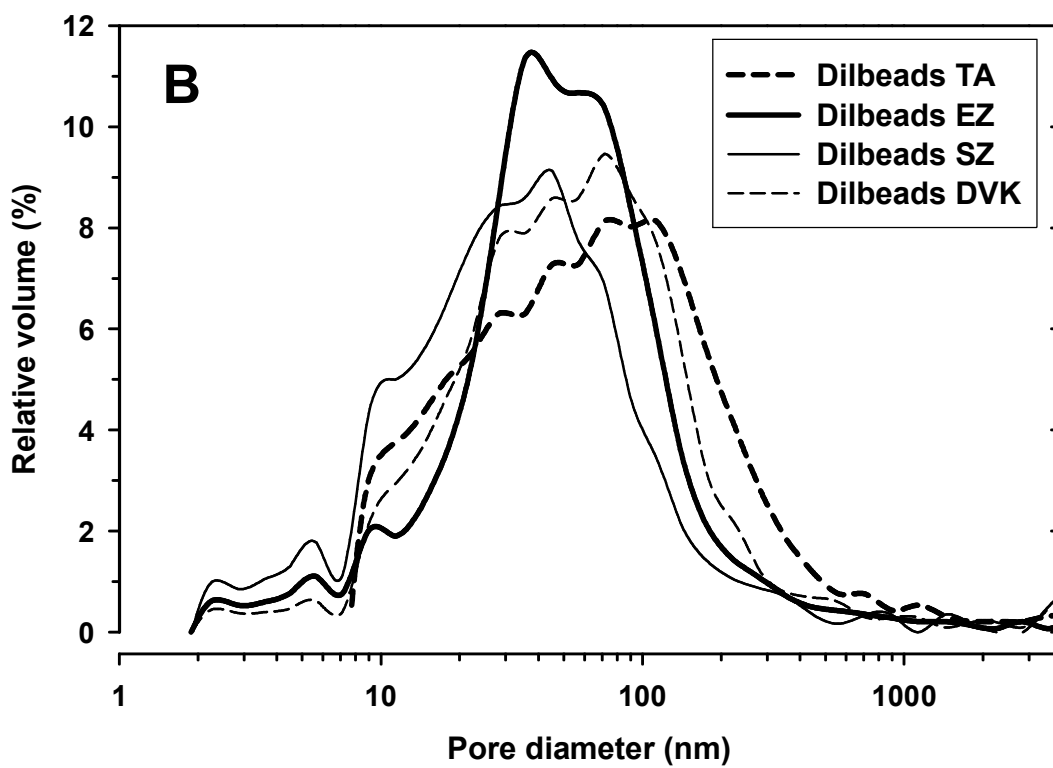
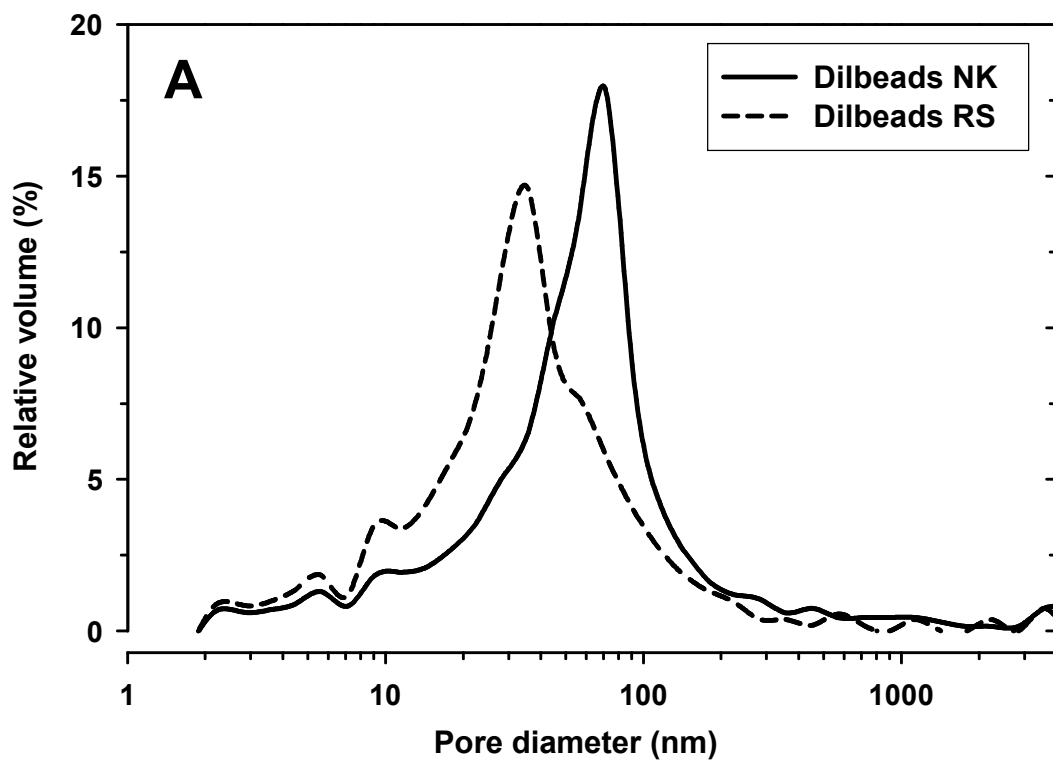


Fig. 3

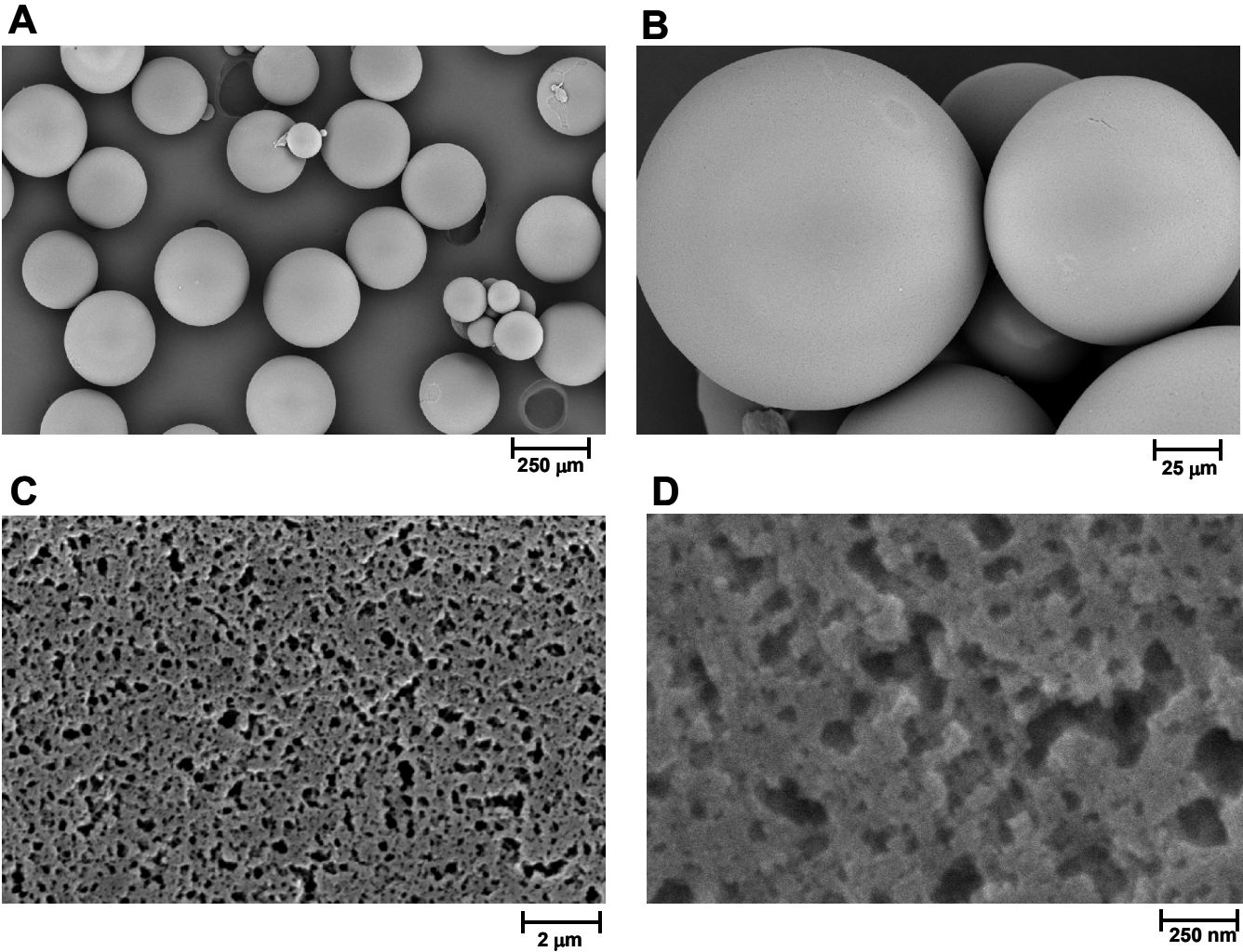


Fig. 4

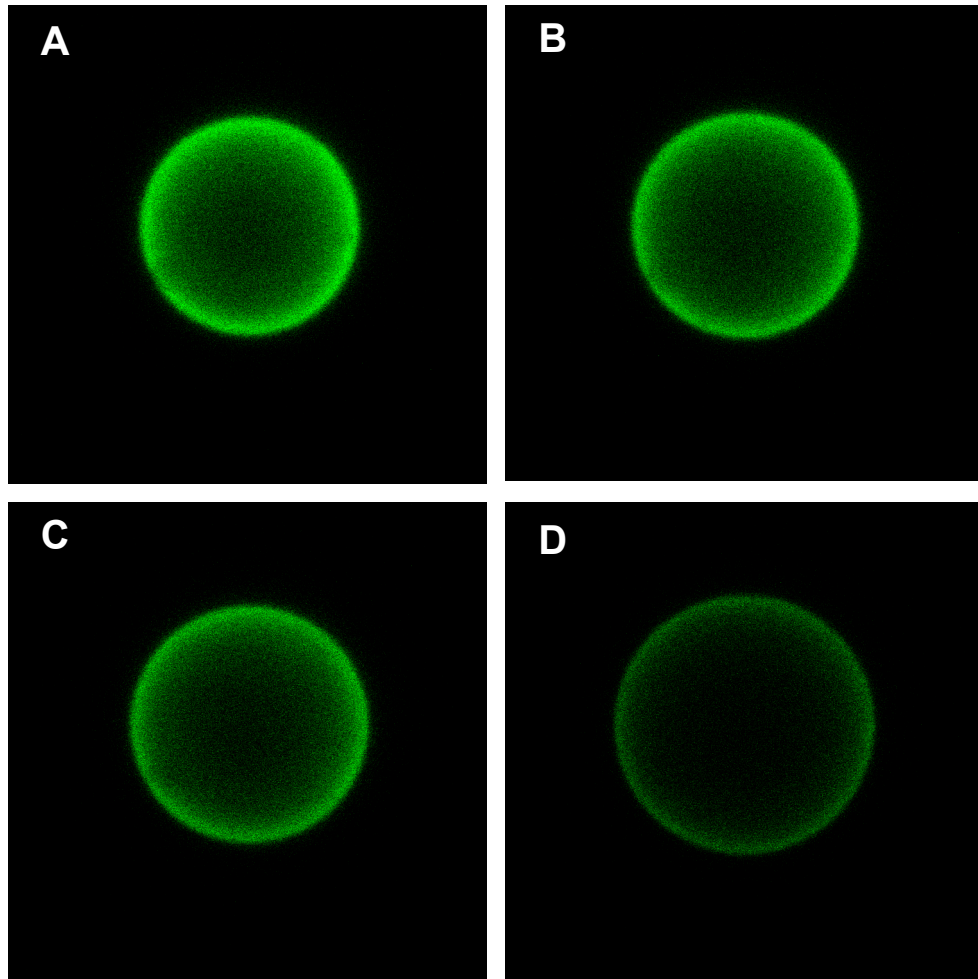


Fig. 5

