1 **Immobilisation and characterisation of biocatalytic co-factor recycling**
2 **enzymes, glucose dehydrogenase and NADH oxidase, on aldehyde functional**
3 **ReSyn™ polymer microspheres**

4

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18

19 **Abstract**

20 The use of enzymes in industrial applications is limited by their instability, cost and
21 difficulty in their recovery and re-use. Immobilisation is a technique which has been
22 shown to alleviate these limitations in biocatalysis. Here we describe the
23 immobilisation of two biocatalytically relevant co-factor recycling enzymes, glucose
24 dehydrogenase (GDH) and NADH oxidase (NOD) on aldehyde functional ReSyn™
25 polymer microspheres with varying functional group densities. The successful
26 immobilisation of the enzymes on this new high capacity microsphere technology
27 resulted in the maintenance of activity of ~40% for GDH and a maximum of 15.4%
28 for NOD. The microsphere variant with highest functional group density of ~3 500

29 µmol.g^{-1} displayed the highest specific activity for the immobilisation of both
30 enzymes at 33.22 U.mg^{-1} and 6.75 U.mg^{-1} for GDH and NOD with respective loading
31 capacities of 51% (0.51 mg.mg^{-1}) and 129% (1.29 mg.mg^{-1}). The immobilised GDH
32 further displayed improved activity in the acidic pH range. Both enzymes displayed
33 improved pH and thermal stability with the most pronounced thermal stability for
34 GDH displayed on ReSyn™ A during temperature incubation at 65 °C with a 13.59
35 fold increase, and NOD with a 2.25-fold improvement at 45 °C on the same
36 microsphere variant. An important finding is the suitability of the microspheres for
37 stabilisation of the multimeric protein GDH.

38

39 **1. Introduction**

40 Biocatalysis is gaining momentum due to the specificity, regio- and enantioselectivity
41 of enzymes over chemical synthetic routes, and are finding application in the
42 production of fine chemicals and pharmaceuticals [1, 2]. However, the realisation of
43 these applications is still limited by their relative instability in desirable reaction
44 systems, including extremes of pH and elevated temperatures [3-7]. A further
45 limitation is their current cost, compounded by difficulties in recovery and re-use of
46 the biocatalyst [8, 9].

47 Immobilisation of the biocatalyst has to an extent alleviated these limitations by
48 reducing the complexity of enzyme recovery and facilitating re-use of the enzyme [5,
49 6, 8, 9]. Immobilisation frequently results in the enhancement of thermal and pH
50 stability, and has previously resulted in improved specificity of the biocatalyst [5, 7-
51 12]. A problem associated with solid supports is their relatively low loading capacity
52 and hence low volumetric activity (high non-catalytic load), resulting in the dilution of
53 specific and volumetric activity of the catalyst [13, 14]. Support based systems that
54 display a high loading capacity is desirable since these can overcome identified
55 limitations of high non-catalytic load such as [15]. The various methods and
56 techniques used to immobilise enzymes is the subject of reviews by Brady and
57 Jordaan [14]; Cao *et al.* [8]; Sheldon [7]; Spahn and Minteer [16]; Tischer and
58 Kasche [17].

59 Oxidoreductases (E.C. 1) are an important class of biocatalyst, and make up about
60 one quarter of all known enzymes [9]. In addition to the various generic limitations of
61 enzymes in large-scale applications, this enzyme classification further requires
62 stoichiometric quantities of expensive nicotinamide co-factors [9, 18]. To reduce the
63 cost associated with the co-factor, this co-substrate is required to be re-used and
64 recycled during biocatalytic reactions [9, 19]. This is suitably achieved through the
65 use of enzymes such as glucose dehydrogenase (GDH, E.C. 1.1.1.47; Fig. 1(a)) and
66 NADH oxidase (E.C. 1.6.3.1; Fig. 2(b)) for recycling these nicotinamide co-factors
67 [20-24].

68 There are few reported cases in the literature relating to the immobilisation of these
69 enzymes, particularly GDH. This may be owing to the proposed difficulties

70 associated with the immobilisation of multimeric enzymes, such as distortion or
71 denaturing, leading to loss in activity [25, 26]. This may be solved through the use of
72 supports which offer multipoint attachment [5]. In this work, we evaluate the
73 immobilisation and characterisation of the biocatalytic co-factor recycling enzymes
74 GDH and NOD, on aldehyde functional ReSyn™ polymer microspheres of varying
75 functional group density. We further characterise the stability of the immobilised
76 enzymes in comparison to the native enzymes in varying pH and temperature
77 conditions.

78

79 **2. Materials and Methods**

80 **2.1. Chemicals and Reagents**

81 Bovine serum albumin (BSA; fraction V; 98%) was purchased from Carl Roth, β-
82 nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide disodium salt
83 hydrate (FAD), potassium dihydrogen orthophosphate (KH_2PO_4), di-potassium
84 hydrogen orthophosphate (K_2HPO_4), sodium chloride (NaCl), hydrochloric acid (HCl;
85 38%), D(+)-glucose, boric acid, citric acid, di-sodium hydrogen orthophosphate
86 (Na_2HPO_4), potassium hydroxide (KOH) and NADH oxidase (NOD - *Bacillus*
87 *licheniformis*) were purchased from Sigma-Aldrich. Glucose dehydrogenase (GDH
88 102 - *Bacillus megaterium*) was purchased from Codexis (USA). β-Nicotinamide
89 adenine dinucleotide free acid Grade I (NAD^+) was obtained from Roche. Quick start
90 Bradford protein assay reagent (including bovine gamma globulin protein standard)
91 was purchased from Bio-Rad Laboratories. Three variants of aldehyde functional
92 ReSyn™ microspheres were gifted by ReSyn™ Biosciences (South Africa). The
93 polymer microspheres varied in their functional group densities of 3500 $\mu mol.g^{-1}$,
94 1200 $\mu mol.g^{-1}$ and 300 $\mu mol.g^{-1}$. The particle size distributions for these
95 microspheres varied from 10.88 ± 3.37 μm, 7.12 ± 0.62 μm and 6.65 ± 1.36 μm
96 respectively. For the purposes of this study these variants were named A, B and C
97 respectively. The microspheres were supplied in 5 ml aqueous suspensions.

98

99 **2.2. Enzyme Preparation**

100 GDH (2.5 ml of 5 $mg.ml^{-1}$) was desalted using PD-10 gel filtration columns (GE
101 Healthcare) according to the manufacturers' protocol to remove preservatives which
102 could potentially interfere with immobilisation. The protein concentration was
103 assayed using Bradford Quick Start protein assay reagent (Bio-Rad) using Bovine
104 Gamma Globulin (BGG) as the standard protein. The GDH enzyme solution was
105 diluted to a final concentration of 1 $mg.ml^{-1}$. NADH oxidase was supplied as a pure
106 protein extract and was therefore not de-salted before immobilisation.

107

108 **2.3. Protein Quantification**

109 Protein was quantified by the method of Bradford [27] using Quick Start Bradford
110 assay reagent (Bio-Rad). Standard curves were generated using BSA as the
111 standard. Briefly, dye reagent, 250 µl, was added to 5 µl of sample and incubated for
112 5 min. The assay was quantified spectrophotometrically at 595nm. All assays were
113 performed in triplicate and data are presented as the mean ± standard deviation.

114

115 **2.4. Enzyme Assays**

116 **2.4.1. Glucose Dehydrogenase Enzyme Assay**

117 Activity for both native and immobilised GDH was determined by following the kinetic
118 reduction of NAD^+ to NADH using spectrophotometric absorbance at 340 nm and
119 monitored on a microtitre plate spectrophotometer (Biotek, PowerWave HT). The
120 assay reagent contained 1 mM NAD^+, 50 mM D(+)-glucose and 50 mM Tris-HCl
121 buffer at pH 8.0. One Unit of GDH was defined as the amount of enzyme required to
122 reduce 1 µmol of NAD^+ per minute at 37 °C. The enzyme reactions contained 5 µl of
123 GDH solution with 195 µl of assay reagent. Microspheres with bound enzyme were
124 washed twice with 1 ml of MilliQ H_2O and resuspended to 100 µl in 20 mM Tris-HCl
125 pH 8.0. Particle preparations were diluted to within the dynamic range of the kinetic
126 assay (as determined using commercial GDH).

127

128 **2.4.2. NADH Oxidase Enzyme Assay**

129 Activity for both native and immobilised NOD was determined by following the kinetic
130 oxidation of NADH to NAD^+ via the same spectrophotometric absorbance used in
131 section 2.4.1. The oxidation of NADH to NAD^+ requires the concomitant conversion
132 of oxygen to hydrogen peroxide/water. Assay reagent contained 1 mM NADH, 0.1
133 mM FAD and 50 mM potassium phosphate buffer pH 7.0. One unit of NADH oxidase
134 was defined as the amount of enzyme required to oxidise 1 μmol of β-NADH per
135 minute at 30 °C. Assay preparations and dilutions are described in 2.4.1 above.
136 Immobilised NOD preparations were made to volume with 20 mM phosphate buffer
137 (KH_2PO_4-K_2HPO_4; pH 7.0). Particle preparations were diluted to within the dynamic
138 range of the kinetic assay (as determined using commercial NOD). No interference
139 from microspheres in the optical pathlength was observed in particle control assays.

140

141 **2.5. Immobilisation of GDH and NOD**

142 The maximum binding capacity of the microspheres was initially determined using
143 BSA as the model protein (Table 1). These values were used to estimate the
144 approximate loading capacity for GDH and NOD (mg protein to per mg microspheres
145 – dry weight). Aqueous suspensions of GDH (1 $mg.ml^{-1}$; pH 8.0) and NOD (0.43
146 $mg.ml^{-1}$; pH 8.0) were used for immobilisation and were loaded onto the
147 microspheres in excess of the estimated binding capacity (20% and 10%
148 respectively) to ensure particle saturation for determination of binding capacity for
149 these enzymes.

150 Immobilisation was conducted by incubation of the particle and enzyme suspension
151 for 60 min at 8 °C with end-over-end mixing at 25 rpm (ELMI Intellimixer). The
152 microspheres were recovered by centrifugation at 6000xg for 5 min and the
153 supernatant assayed for unbound protein using the Bio-Rad assay mentioned above.
154 The particles were washed once with 1 ml of MilliQ water. The protein content of the
155 immobilisation and wash supernatants were quantified. To determine the extent of
156 non-specific interaction, the microspheres were further washed with 2 M NaCl to
157 remove non-covalently linked protein, with subsequent protein quantification after
158 microsphere recovery by centrifugation. No loss in enzyme activity was observed for

159 free NOD or GDH incubated in the immobilisation conditions. Extended incubation
160 times of up to 24 hours did not result in higher immobilisation capacities.

161 For subsequent immobilisations, the experimentally determined covalent capacity for
162 GDH and NOD was used for microsphere loading. All experimentation was
163 performed in triplicate and data presented as the mean ± standard deviations. The
164 immobilisation capacities of the solid supports are presented as weight of
165 immobilised enzyme per unit solid support (dry weight).

166

167 **2.6. Enzyme Activity Maintenance**

168 The enzyme activity maintenance was defined as the percentage of the residual
169 activity of the immobilised enzyme, compared to the total enzyme activity loaded
170 (calculated as the difference between the enzyme activity used for immobilisation
171 and the residual activity in solution after removal of the microspheres). These values
172 are expressed as a percentage (Fig. 2). The activity assays for GDH and NOD were
173 performed as described in 2.4.1 and 2.4.2. Specific activities of immobilised
174 enzymes are presented as enzyme activity retained per unit mass of enzyme, as well
175 as per unit mass of enzyme and solid support, including the mass of the non-
176 catalytic solid support to give an indication of the volumetric activity.

177

178 **2.7. pH Profiling**

179 The pH profile assays were performed using the methods described above in 2.4.1
180 and 2.4.2. However, the standard assay buffers were replaced with a universal buffer
181 containing 50 mM phosphate (Na_2HPO_4), 50 mM Boric Acid, 33 mM Citric Acid and
182 50 mM Tris adjusted to pH values between 4-10 using HCl or KOH [28].

183

184 **2.8. Thermal Stability**

185 Thermal stability was determined by incubation of the free and immobilised GDH
186 preparations at temperatures from 50 to 65 °C with 5 °C increments while NOD was

incubated at 35 and 40 °C. Enzyme activities were assayed initially at 5 min intervals using the methods and conditions described in 2.4.1 and 2.4.2. The time interval was extended for samples displaying high stability. Samples were removed and allowed to cool to room temperature before assaying.

2.9. pH Stability

pH stability was determined by incubation of the free and immobilised GDH and NOD enzyme preparations in universal buffer between pH 2.5 and 10.0. The standard activity assays were performed as described above (2.4.1 and 2.4.2).

3. Results

3.1. Immobilisation of GDH and NOD

The immobilisation of enzymes using aldehyde functional supports occurs through Schiff-base bond formation with the primary amines of the proteins [5]. The immobilisation procedure is performed at pH 8.0 to ensure reactivity of the nucleophilic primary amines with aldehydes available on the microspheres [5]. The microsphere preparations had varying capacities for biomolecule immobilisation, with direct proportionality to the functional group density. This trend was observed when using BSA and NOD for immobilisation (Table 1). However GDH did not appear to follow this trend with ReSyn™ B binding the highest quantity enzyme. This may have been due to a difference in the internal environment and or porosity of this product, which may have induced changes in the nature and/or intensity of interactions between the enzyme and support [29]. Reduction of the resultant Schiff base bonds with cyanoborohydride resulted in a loss of 77% of the enzyme activity (GDH). This may have been owing to the ability of reducing agents to reduce disulphide bridges [30]

214 ## 3.2. Enzyme Activity Maintenance and Specific Activity of Immobilised
215 GDH and NOD

216 It is important to determine the maintenance of catalytic activity by the immobilised
217 enzyme when evaluating a technique for immobilisation. The maintenance in activity
218 of GDH and NOD immobilised on the various preparations is shown in figure 2. The
219 enzyme activity maintenance for GDH was generally in the region of 40% with little
220 variance between the microsphere preparations (Fig. 2). Immobilised preparations of
221 NOD displayed activity maintenance with a higher degree of variance ranging from
222 12.3 to 23.6% (Fig. 2).

223 The specific activity of an immobilised enzyme is influenced by the nature of the
224 support material, enzyme loading capacity, and maintenance in enzyme activity [13].
225 The specific activity of immobilised GDH preparations ($U.mg^{-1}$ enzyme) was lowest
226 on ReSyn™ C at 42.19 $U.mg^{-1}$, while ReSyn™ A and B had higher specific activities
227 of 54.73 and 49.09 $U.mg^{-1}$ respectively (Table 2). NOD immobilised on ReSyn™ A
228 had the highest specific activity of 8.09 $U.mg^{-1}$ followed by ReSyn™ C with 6.33
229 $U.mg^{-1}$ and ReSyn™ B the lowest at 4.21 $U.mg^{-1}$ (Table 2). To illustrate the high
230 volumetric activity, the data is further represented as specific activity inclusive of the
231 non-catalytic function (microsphere weight).

232

233 ## 3.3. pH Profiling

234 The pH profile for the free and immobilised GDH and NOD is illustrated in figure 3.
235 All GDH preparations, free and immobilised, showed optimal activity at pH 8.0, while
236 NOD displayed optimal activity at pH 7.0. GDH immobilised on ReSyn™ A exhibited
237 a broader pH profile in the acidic range (pH 5.0-7.0), indicated by a ~2-fold increase
238 in activity in this range compared to that of the native enzyme (Fig. 3). ReSyn™ B
239 and C preparations exhibited the same pH profile as the native enzyme (Fig. 3).
240 There was no shift or notable difference in the pH profile of immobilised NOD
241 preparations when compared to the profile of native NOD (Fig. 3 insert).

242

243 ## 3.4. Thermal and pH Stability

244 GDH immobilised on ReSyn™ A displayed the highest thermal stability with half-life
245 improvements of 2.89, 6.82, 9.21 and 13.59-fold at 50, 55, 60 and 65 °C respectively
246 over that of the free enzyme (Fig. 4), ReSyn™ B and C GDH also displayed
247 improved thermal stability, albeit less pronounced (Fig. 4). The enzyme was further
248 stabilised against denaturation under acidic conditions (Table 3a) with ReSyn™ A
249 again displaying the highest stability with a 2.37 fold improvement in half-life at pH
250 3.5, and approximately 2 fold at pH 2.5 and 3 (Table 3a). GDH immobilised on
251 ReSyn™ B and C again displayed reduced pH stability compared to ReSyn™ A.
252 These findings appear to confirm that functional stability is proportional to the
253 reactive group density used for immobilisation [5, 31].

254 Incubation of immobilised NOD at elevated temperatures indicated that only
255 ReSyn™ A and B provided improved thermal and pH stability. ReSyn™ C did not
256 display any improved thermal stability over the free enzyme during incubation at 40
257 and 45 °C (Table 3b). ReSyn™ A provided improved protection against thermal
258 denaturation of NOD with improvements at 40 °C (1.87 fold) and 45 °C (2.25 fold)
259 respectively. However, this did not coincide with pH stability. ReSyn™ B provided
260 the best enhancement in pH stability, increasing the half-life of NOD activity from 14
261 to 141 minutes at pH 3.0. ReSyn™ A and C appeared to marginally enhance the
262 stability at pH 3.0 (Table 3b).

263

264 **4. Discussion**

265 The claimed functional group densities ranging from 300 to 3500 μmoles.g^{-1}
266 (ReSyn™ Biosciences) and the fibrous interpenetrating network of ReSyn™
267 microspheres [14] are proposed to provide an increased surface area resulting in a
268 high binding capacity (www.resynbio.com). This feature was witnessed for the
269 immobilisation of the standard control protein (BSA), GDH and NOD. The results
270 presented here for ReSyn™ microspheres far exceed previously reported literature.
271 Covalent immobilisation of GDH on controlled pore silica by Baron *et al.* [20]
272 demonstrated a maximum binding capacity of 0.4 mg.g^{-1} (Table 4) while here we
273 report a loading capacity of 820 mg.g^{-1} by ReSyn™ B. The lack of literature on GDH
274 immobilisation is surprising considering its value as a co-factor recycling agent in

275 biocatalysis, but may be associated with the challenges of immobilising multimeric
276 enzymes [25, 26]. The ReSyn™ microspheres further resulted in improved enzyme
277 activity maintenance of 41.5% for ReSyn™ A, as compared to the values reported by
278 Baron *et al.* [20] of 1.15% (Table 4). The resultant specific activities of ReSyn™ A
279 immobilised GDH was calculated as 54.73 $U.mg^{-1}$ (or 33.22 $U.mg^{-1}$ inclusive of
280 microsphere support) through a combination of relatively high enzyme activity
281 maintenance and the high capacity of the microspheres. In comparison, the specific
282 activity for the immobilisation on silica support was previously reported as 2.74
283 $U.mg^{-1}$ (enzyme only) (Table 4).

284 The binding capacity achieved for the immobilisation of NOD on aldehyde functional
285 ReSyn™ also exceeded previously reported results. Sanjust *et al.* [32] reported a
286 maximum capacity of 17 $mg.g^{-1}$ for the covalent immobilisation of NOD on both
287 cyanogen bromide activated PVA beads and Sephacryl S-200 HR treated with 2,4,6-
288 Trichloro-1,3,5-triazine (TCT) (Table 4) while in this study ReSyn™ A achieved 1 288
289 $mg.g^{-1}$. More recently, NOD was immobilised on single-walled carbon nanotubes
290 (SWCNTs) with a resultant capacity of 470 $mg.g^{-1}$ [33]. Although this is lower than
291 what was achieved on ReSyn™, the SWCNT achieved an enzyme activity
292 maintenance of 92% ([33]; Table 4) while the highest achieved for ReSyn™ was
293 23.6 %. This comparatively low maintenance in enzyme activity maintenance
294 resulted in a specific activity of 8.09 as compared to 52.4 $U.mg^{-1}$ obtained with
295 SWCNT's.

296 Apart from the well understood factors that may have affected enzyme activity
297 maintenance of NOD and GDH such as steric hindrance [31, 34, 35]; interaction and
298 orientation on the support and change in the micro-environment upon immobilisation
299 [8, 13]; the high capacity of ReSyn™ microspheres has a high potential for mass
300 transfer limitations of substrate and/or products [13, 29, 34, 36, 37].

301 More specifically, for GDH the loss in activity may be as a direct result of the active
302 site lysine residue (Lys-201) taking part in the immobilisation process, an integral
303 amino acid required for activity and/or ligand binding in the enzyme [38]. ReSyn™
304 microspheres are loosely-linked polyethyleneimine matrices (www.resynbio.com).
305 Interestingly, PEI has been shown to play a role in the stabilisation of oxygen-

306 sensitive nitrilases, which was attributed to the low permeability and reduced
307 solubility of oxygen in this polymer [39]. Since NOD utilises oxygen as a substrate,
308 this property of polyethyleneimine may have resulted in the relatively low enzyme
309 activity maintenance of 23.6%.

310 It is well known that the microenvironment of the immobilisation matrix can result in a
311 shift in the optimal pH of an enzyme [40, 41]. In this experimentation there appeared
312 to be no change in the optimum pH of either of the immobilised enzymes (Fig. 3).
313 However, ReSyn™ A immobilised GDH displayed enhanced activity in the acidic pH
314 range, potentially enabling applications in these conditions where this enzyme is
315 currently not suitable. An example of where this feature may enhance applicability of
316 this enzyme is the synthesis of gluconic acid in the food industry [42].

317

318 **5. Conclusions**

319 In general, ReSyn™ immobilised enzymes displayed improved stability at elevated
320 temperature and acidic pH conditions. For immobilised enzyme preparations the
321 highest thermal stability was displayed on ReSyn™ A microspheres having the
322 highest functional group density of 3 500 $\mu mol.g^{-1}$. This improved stability of
323 immobilised enzymes is proposed to result from “rigidification” of the enzyme’s three-
324 dimensional structure which provides resistance to conformational change induced
325 by environmental conditions [5, 31, 43, 44], while the degree of multipoint attachment
326 (a feature of the available functional group density) has been proposed to be directly
327 proportional to improvement in stability [5, 31]. The results presented here are in
328 good agreement with this hypothesis. Stability in immobilised enzymes may also be
329 enhanced by the micro-environment provided by the support [5, 12, 31]. The
330 proposed structure of the microspheres, i.e. a porous loosely-linked polymer matrix,
331 appears to allow shielding from the harsh external environment. The results
332 demonstrate a microsphere with an exceptional high protein binding capacity with
333 maximum loading of 50% achieved for GDH and 129% for NOD. Of particular
334 interest is the immobilisation of the multimeric enzyme GDH since multi-subunit
335 enzymes are notoriously difficult to immobilise [25, 26]. The successful

336 immobilisation of these enzymes may be used to enhance their suitability as co-
337 factor recycling agents in biocatalytic applications.

338

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342

343

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455 prevent subunit dissociation. Enzyme Microb Technol. 2009;45:405-18.

456

457 **List of Tables**

458

459 **Table 1**: Binding capacity of GDH and NOD immobilised on various ReSyn™
460 preparations

ReSyn™ Preparation	Microsphere dry weight ($mg.ml^{-1}$)	Binding Capacity ($mg.g^{-1}$ $_{dry\ support}$)		
		BSA	GDH	NOD
A	8.56 ± 0.61	1 711 ± 20	508 ± 2	1 288 ± 1
B	6.35 ± 0.35	950 ± 40	820 ± 2	837 ± 3
C	7.41 ± 0.40	292 ± 30	163 ± 30	220 ± 12

461

462

463 **Table 2**: Specific activities of GDH and NOD calculated including and excluding the
464 non-catalytic load (support)

ReSyn™ Preparation	Specific activity ($U.mg^{-1}$ $_{enzyme}$)		Specific activity ($U.mg^{-1}$ $_{support\ and\ enzyme}$)	
	GDH	NOD	GDH	NOD
A	54.73 ± 0.06	8.09 ± 0.73	33.22 ± 0.01	6.75 ± 0.03
B	42.19 ± 0.23	4.21 ± 0.08	33.01 ± 0.07	1.80 ± 0.05
C	49.09 ± 0.22	6.33 ± 0.06	12.76 ± 0.03	4.95 ± 0.03

465
466 The specific activities of commercial enzymes were determined as 116 $U.mg^{-1}$ for GDH and 34.2
467 $U.mg^{-1}$ for NOD.

468

469 **Table 3a**: Half-life values indicating pH stability of free and immobilised GDH

Preparation	pH stability ($t_{50\%}$-min)		
	2.5	3	3.5
Native GDH	8 ± 1.61	27 ± 1.02	140 ± 2.10
ReSyn™ A-GDH	18 ± 2.10	50 ± 2.32	332 ± 2.87
ReSyn™ B-GDH	7 ± 1.59	34 ± 1.70	274 ± 1.01
ReSyn™ C-GDH	7 ± 1.81	28 ± 0.80	239 ± 1.21

470

471

472 **Table 3b**: Half-life values for free and immobilised NOD obtained from thermal and
473 pH stability assays

	Thermal stability ($t_{50\%}$ - min)		pH stability ($t_{50\%}$ - min)		
Preparation	**40 °C**	**45 °C**	**3.0**	**4.0**	**10.0**
Native NOD	47 ± 1.77	40 ± 1.84	14 ± 1.74	114 ± 0.61	116 ± 0.47
ReSyn™ A-NOD	88 ± 1.02	90 ± 2.38	48 ± 0.32	173 ± 0.45	164 ± 0.406
ReSyn™ B-NOD	44 ± 1.70	31 ± 1.81	141 ± 3.53	265 ± 1.16	233 ± 0.79
ReSyn™ C-NOD	43 ± 0.34	35 ± 0.93	43 ± 2.47	113 ± 1.94	147 ± 1.52

474

475

476 **Table 4**: Covalent immobilisation of GDH and NOD on various supports

Enzyme	Immobilisation Support	Binding Capacity ($mg.g^{-1}{}_{support}$)	Maintenance in Activity (%)	Specific Activity ($U.mg^{-1}{}_{enzyme}$)	Reference
GDH	CPS[a]-500	0.4	1.15	2.74	[20]
	ReSyn™ A	508	41.5	54.73	Current Study
NOD	Cross-linked PVA (CNBr[b])	16.5	21	4.3	[32]
	Sephacryl S-200 HR (TCT[c])	16.5	23	4.9	[32]
	Glutarylaminopropyl-PVA[d] (NHS[e])	16	38	8.3	[32]
	SWCNTs[f]	470	92	54.2	[33]
	ReSyn™ A	1 288	23.6	8.09	Current Study

477

478 a – controlled pore silica, b – cyanogen bromide, c – 2,4,6-Trichloro-1,3,5-triazine, d – polyvinyl
479 alcohol, e – N-hydroxy-succinimide, f – single walled carbon nanotubes

480

481

482 **List of Figures**

483

484 **Fig. 1**: Enzymatic reactions for (a) glucose dehydrogenase and (b) NADH oxidase
485 indicating the utilisation and regeneration of the nicotinamide co-factors.

486

487 **Fig. 2**. Average maintenance in activity (as defined in the text) displayed by GDH (■)
488 and NOD (■) immobilised on various ReSyn™ preparations. The data are presented
489 as the mean of triplicate data ± the standard deviation.

490

491 **Fig. 3**. pH profile of native GDH (─●─) and GDH immobilised on ReSyn™ A (- -■- -),
492 B (- ·▲· -) and C (·····◆·····). Optimal activity was displayed at pH 8.0. ReSyn™ B-GDH
493 displayed a broader pH profile with increased activity over the acidic range. **Insert:**
494 Free (─●─) and immobilised NOD on ReSyn™ A (- -■- -), B (- ·▲· -) and C (·····◆·····),
495 optimal activity was displayed at pH 7.0 for all preparations. The data are presented
496 as the mean of triplicate data ± the standard deviation.

497

498 **Fig. 4**. Temperature stability of non-immobilised (●) and GDH immobilised on
499 ReSyn™ A (▪), B (▲) and C (♦) during incubations at 50, 55, 60 and 65 °C. **Insert:**
500 Half-life plots of native GDH (─●─) and GDH immobilised on various ReSyn™
501 preparations A (- -■- -), B (- ·▲· -) and C (·····◆·····) at 50, 55, 60 and 65°C.

502