Studies on Conformation of Soluble and Immobilized Enzymes Using Differential Scanning Calorimetry. 1. Thermal Stability of Nicotinamide Adenine Dinucleotide Dependent Dehydrogenases†

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ABSTRACT: The technique of differential scanning calorimetry (DSC) has been applied to the study of temperature-induced irreversible denaturation and thus to the heat stability of soluble and Sepharose-bound liver alcohol dehydrogenase (LADH, EC 1.1.1.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) in the presence of various coenzymes or coenzyme fragments. The transition temperature (T_{tr}) of 82.5 °C obtained for soluble LADH was increased by 12.5 °C in the presence of a saturating concentration of NADH. In the presence of NAD^+, T_{tr} increased by 8.5 °C, whereas ADP-ribose and AMP caused an increase in T_{tr} of only 2 and 1 °C, respectively. The T_{tr} of 85.5 °C obtained for Sepharose-bound LADH was increased by about 12 °C after the addition of free NADH. However, when the enzyme was immobilized simultaneously with a NADH analogue (which also binds to the matrix), a broad endotherm with a T_{tr} of 91.5 °C was obtained, indicating the presence of immobilized enzyme molecules both with, and without, associated NADH. Corresponding increases in heat stability were observed for LDH in solution in the presence of NADH, NAD^+, and AMP, leading to increases in T_{tr} from 72 to 79.5 and 74 and 73 °C, respectively. The addition of pyruvate and NAD^+ to the enzyme to form an abortive ternary complex led to the same stabilization as that observed with NADH, attendant with a large increase in the enthalpy of transition, ΔH_{tr}. In these studies the technique of DSC was utilized because it is applicable both to soluble and immobilized enzymes and (1) provides rapid information about T_{tr} and thus the thermal stability of enzymes, (2) different energetic states of an enzyme molecule can be identified, and (3) an overall picture of the thermal process is rapidly obtained.

The technique of differential scanning calorimetry (DSC)[1] has only recently been developed, and its application to the study of biopolymers (Privalov, 1974) is still in its infancy. The DSC studies reported thus far hold great promise and it is expected that further important contributions will be made in analogy to those reported in the study of a variety of biochemical questions such as protein denaturation (Beck et al., 1965; Delben et al., 1969; Tsong et al., 1970; Privalov and Khechinashvili, 1974; Hägerdal and Martens, 1977) and the nature of association between macromolecules (Donovan and Beardslee, 1975) or between the protein avidin and biotin (Donovan and Ross, 1973).

By this method endo- and/or exothermic processes which take place on heating a sample can be followed, providing information on various thermodynamic parameters. In addition, and of particular relevance to the present study, the transition temperature, T_{tr}, is readily obtained from the thermogram.

The recent interest in the application of enzymes, usually in an immobilized state, to analysis, medicine, and technology (Vieth and Venkatasubramanian, 1973, 1974; Pye and Wingard, 1974; Mosbach, 1976) has accentuated the need for enzyme preparations of high stability, including heat stability. Usually stability tests are carried out by laborious heat treatment of the enzyme at various temperatures and lengths of incubation followed by activity tests. An alternative method to heat stability tests would involve direct calorimetric measurements using an absolute calorimeter. Such measurements, however, are both time consuming and require relatively large amounts of protein. The potential of differential scanning calorimetry was therefore investigated as a rapid and inexpensive alternative for determination of heat stability. Since the matrices of immobilized enzyme preparations were not expected to interfere, this technique was also applied to the study of such preparations.

In addition, it was expected that a more detailed picture of the heat denaturation process for soluble as well as for immobilized enzyme might be obtained by employing DSC. Moreover, various states of biological significance, such as ligand bonded forms of enzymes and different conformational states of immobilized enzymes could possibly be identified by this technique.

Until now, in the reported studies concerning the principles of stabilization of proteins using the DSC technique, the well-characterized monomeric enzymes ribonuclease A (Beck et al., 1965; Delben et al., 1969; Tsong et al., 1970; Privalov and Khechinashvili, 1974), α-chymotrypsin, cytochrome *c*, myoglobin (Privalov and Khechinashvili, 1974), and trypsin (Donovan and Beardslee, 1975) in soluble form have been used.

In this paper we report on DSC studies of oligomeric and structurally more complex molecules such as liver alcohol dehydrogenase, LADH (MW 80 000) and lactate dehydrogenase, LDH (MW 140 000), in soluble form and covalently bound to a Sepharose matrix. Primary emphasis has been put on the possible heat-stabilizing effects of their corresponding coenzyme and coenzyme fragments, which is indicated by larger or smaller displacements of the endotherm compared with the apoenzyme. Moreover, the thermal behavior of the immobilized enzyme and its ability to bind a coenzyme molecule was studied and compared with the behavior of the soluble

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[1] Abbreviations used: LADH, liver alcohol dehydrogenase (EC 1.1.1.1); LDH, lactate dehydrogenase (EC 1.1.1.27); DSC, differential scanning calorimetry; T_{tr}, transition temperature; ΔH_{tr}, enthalpy of transition; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: The Transition Temperature of Soluble and Sepharose CL 4B Bound LADH and LDH in Presence or Absence of Various Coenzymes or Coenzyme Fragments.[a]

	Liver Alcohol Dehydrogenase					Lactate Dehydrogenase				
	Soluble			Immobilized		Soluble			Immobilized	
Nucleotide	Nucleotide Concn (mM)	T_{tr} (°C)	ΔH_{tr} (kcal/mol)	Nucleotide Concn (mM)	T_{tr} (°C)	Nucleotide Concn (mM)	T_{tr} (°C)	ΔH_{tr} (kcal/mol)	Nucleotide Concn (mM)	T_{tr} (°C)
		82.5	350		85.5		72	490		74.5
AMP	17	83.5	344			33	73	485		
AMP + NMN	17 + 17	83.5	350							
ADP-ribose	17	84.5	361							
NAD^+	17	91	367			33	74	490		
NAD^+ + pyruvate (90 mM)						33	79.5	664		
NADH	17	95	402	17	98	33	79.5	572	33	82.5

[a] The enthalpies of transition are given for soluble LADH and LDH at pH 8.1 and in the presence of various nucleotides.

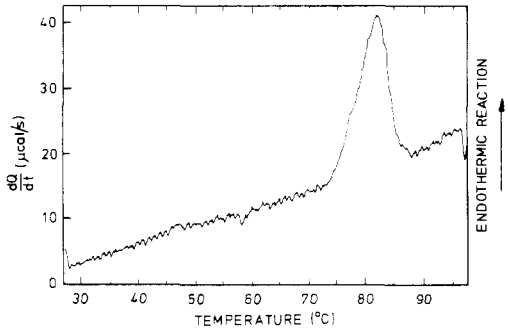

FIGURE 1: A complete tracing of the thermogram obtained for soluble LADH, pH 8.1. The amount of protein was about 200 μg. Heating rate: 10 °C/min.

enzyme. In the following paper (Koch-Schmidt and Mosbach, 1977), DSC has been used to characterize immobilized enzymes. In this investigation the effect of the number of covalent linkages between the enzyme molecule and the Sepharose matrix on the specific activity and heat stability has been evaluated.

Experimental Section

Materials

Alcohol dehydrogenase (horse liver, 2.7 U/mg) and lactate dehydrogenase (pig heart, 360 U/mg) were obtained from Boehringer (Mannheim, West Germany), β-NAD^+ (grade III), β-NADH (grade III), ADP-ribose, AMP (type II), β-nicotinamide mononucleotide, NMN, and pyruvate were from Sigma (St. Louis, Mo.), Sepharose CL 4B was from Pharmacia (Uppsala, Sweden), and cyanogen bromide was from Fluka AG (Buchs SG, Switzerland). All other reagents used were of analytical grade.

N^6-[(6-Aminohexyl)carbamoylmethyl]NADH (prepared according to Lindberg et al., 1974) was synthesized by Dr. P.-O. Larsson of our department.

Methods

1. Preparation of Immobilized LADH and LDH. The enzymes were coupled to Sepharose CL 4B by the cyanogen bromide method (Axén et al., 1967) modified as follows.

Sepharose CL 4B was washed and sucked on a glass filter. Two grams of the gel were added to 10 mL of a cold (4 °C) water solution containing 200 mg of CNBr. The activation proceeded for 5 min at 4 °C and pH 10.8 by titration using 1 M NaOH. Activated beads were washed with 1 L of cold 0.1 M $NaHCO_3$, pH 8.4. Activated beads (500 mg) were transferred to 2 mL of a 0.1 M $NaHCO_3$ solution (pH 8.4) containing about 10 mg of the enzyme, which had previously been dialyzed against 2 × 250 mL of 0.1 M $NaHCO_3$ for 4 h at 4 °C.

When binding LADH simultaneously with N^6-[(6-aminohexyl)carbamoylmethyl]NADH, the enzyme (10 mg) and the cofactor analogue (5 mM) were allowed to equilibrate in 2 mL of 0.1 M $NaHCO_3$, pH 8.4, for half an hour at 4 °C before the addition of 500 mg of activated beads was made.

2. Thermal Analysis of Soluble and Immobilized LADH and LDH in Presence or Absence of NAD(H) and Its Fragments. Thermal analysis of soluble and immobilized enzymes was carried out using a DSC-2 differential scanning calorimeter (Perkin-Elmer) equipped with a cooling system. Aluminum pans made for water solutions and holding up to 18 μL of liquid were used exclusively. The pans were pressure sealed and weighed before heating.

In the analysis of soluble enzyme, the sample pan was filled with 15 μL of the enzyme solution (approximately 1% which had been previously dialyzed against 0.1 M Tris buffer, 0.1 M in NaCl, pH 8.1) both with and without saturating concentrations of coenzyme; see Table I. In the case of immobilized enzyme, the pan was filled with 7–12 mg of well-sucked gel, after which an additional 5 μL of 0.1 M Tris buffer–0.1 M in NaCl, pH 8.1, was added in order to remove air bubbles in the sample. In all the analyses the reference pan contained 15 μL of the buffer solution. Also in the cases where the sample contained a matrix or a cofactor, these were omitted in the reference as neither showed any transition in the temperature interval studied.

The thermograms were run from about 290 to 380 K at the highest sensitivity (0.1 mcal/s) using a heating rate of 10 °C/min. Full scale over the recorder corresponded to 0.05 mcal/s and the paper speed was 40 mm/min. In order to provide information concerning possible drift and noise in the instrument, a complete tracing of the thermogram obtained for soluble LADH (heated from 290 to 369 K) is given in Figure 1.

The transition temperature, T_{tr}, is defined as the temperature at the peak of the endotherm.

ΔH_{tr}/mole values of the soluble enzymes were obtained from the areas under the endotherm peak and using protein concentrations based on absorption values at 280 nm using $\epsilon_{280} = 3.64 \times 10^4\ cm^{-1}\ M^{-1}$ for LADH and $\epsilon_{280} = 1.9 \times 10^5\ cm^{-1}$

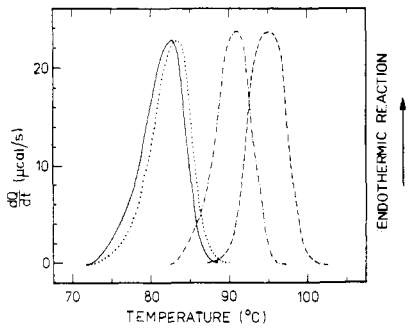

FIGURE 2: Denaturation thermograms at pH 8.1 of solutions of LADH (—), LADH + 17 mM AMP (· · ·), LADH + 17 mM NAD^+ (- · · -), and LADH + 17 mM NADH (- - -). The amount of protein in each run was about 200 μg. Heating rate: 10 °C/min.

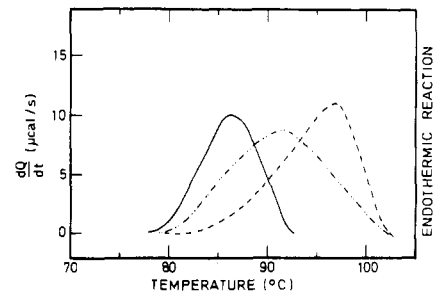

FIGURE 3: Denaturation thermograms at pH 8.1 of Sepharose-bound LADH (—), Sepharose-bound LADH in presence of 17 mM free NADH (- - -), and Sepharose-bound LADH–NADH analogue (- · · · -). Heating rate: 10 °C/min.

M^{-1} for LDH. The temperatures were reproducible to within 1 °C and the peak area was to within 7%.

Results and Discussion

In the first part of this investigation, studies were made on the temperature-induced irreversible denaturation of soluble and Sepharose-bound liver alcohol dehydrogenase, LADH, in the presence or absence of various adenine nucleotides using DSC. The heat stability of various enzyme states is reflected by T_{tr} and can be compared under identical conditions of pH, heating rate, ionic strength, etc.

As seen in Figure 2, a T_{tr} of 82.5 °C is obtained for soluble LADH. The addition of saturating concentration of NADH (Table I) causes a remarkable heat stabilization of the enzyme of 12.5 °C, which is in accordance with previous observations on the stabilization of LADH by its coenzymes, determined as the remaining enzymic activity after heating the enzyme at 75 °C for various lengths of time (Theorell and Tatemoto, 1971). The addition of other adenine nucleotides to soluble LADH showed that NAD^+ enhanced T_{tr} by about 8.5 °C, while AMP caused only 1 °C increase as seen in Figure 2 and Table I. No further displacement was found after the addition of NMN to the LADH–AMP complex (Table I). In this context it should be mentioned that the same relative positions of the T_{tr} values were obtained independent of the scanning rate used. However, owing to the time lag of the apparatus, the individual T_{tr} value decreases somewhat (1–2 °C) with a decrease in the scanning rate. In order to investigate whether these displacements could be correlated to structural changes of the LADH-molecule taking place in the presence of various nucleotides, thermal analysis was also carried out on LADH to which ADP-ribose had been added. This coenzyme analogue binds to the LADH molecule in a manner similar to NAD^+, but does not induce any conformational changes of LADH (Brändén et al., 1973). With the DSC technique we observed in this case an increase in T_{tr} of only 1.5 to 2 °C compared with the free enzyme (Table I) which suggests that in fact there is such a correlation.

Reported data provide evidence of large conformational changes when NADH and NAD^+ are bound to LADH, while no such changes have been reported for ADP-ribose and AMP (Brändén et al., 1973; Eklund et al., 1974). Thus it appears highly likely that the observed increase in transition temperature compared with the apoenzyme is a consequence of the formation of an energetically more stable conformation of the enzyme molecule when it is associated with a coenzyme.

The actual ligand concentration (17 mM) used in the experiments (Table I) displaces the equilibrium far in the direction of the enzyme–ligand complex, as the K_{diss} values found in the literature for the binary complexes of LADH with various adenine nucleotides at pH 8.0 are: LADH–AMP, 90 μM (Gunnarsson and Pettersson, 1974); LADH–ADP-ribose, 21 μM (Yonetani, 1963); LADH–NAD^+, 141 μM, and LADH–NADH, 0.28 μM (Theorell and McKinley-McKee, 1961).

The ΔH_{tr} values (Table I) of the various binary complexes did not differ significantly from those of free LADH, except for a small increase (about 10%) for the LADH–NADH complex, which, however, can be explained by the tendency of ΔH_{tr} to increase with T_{tr} as observed by Privalov and Khechinashvili (1974). The ΔH_{tr} values obtained were independent of scanning rate.

When immobilized to a Sepharose matrix, LADH showed 2–3 °C increase in T_{tr} compared with the soluble enzyme (cf. Koch-Schmidt and Mosbach, 1977). The presence of the matrix itself does not cause this displacement as soluble enzyme mixed with Sepharose beads showed the same original T_{tr}. It is interesting to note that immobilized enzyme molecules which exhibit thermal transition (Figure 3) are also capable of binding NADH molecules and can thus be heat stabilized by about 12 °C in the same way as the soluble enzyme. This is demonstrated by the fact that there is only one peak seen when free NADH is added to the immobilized LADH preparation.

When LADH is immobilized on a Sepharose matrix, the preparation obtained does not require externally added NADH for its activity if the procedure is carried out in the presence of excess of the NADH analogue, N^6-[(6-aminohexyl)carbamoylmethyl]NADH (Gestrelius et al., 1975). The latter is covalently bound to the matrix through its terminal amino group. The finding that no externally added NADH is required has been interpreted as due to the presence of enzyme–coenzyme complexes. Both enzyme and coenzyme are covalently bound to the matrix so that the coenzyme is oriented near or in the active site of the enzyme. Further addition of free NADH does, however, increase activity of such a preparation. It has therefore been concluded that not all enzyme molecules or subunits of the dimeric enzyme show this optimal orientation of coenzyme to enzyme. The endotherm obtained for such an enzyme–coenzyme preparation, as seen in Figure 3, is rather broad and shows a T_{tr} of 91.5 °C. The thermogram overlaps those obtained for immobilized LADH when heated in the presence or absence of externally added, unmodified NADH. This overlap is in accord with the heterogeneous nature of such preparations.

Corresponding results were obtained for lactate dehydrogenase, LDH, both soluble and immobilized to Sepharose CL

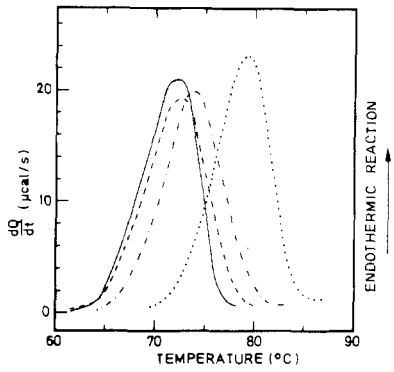

FIGURE 4: Denaturation thermograms at pH 8.1 of solutions of LDH (—), LDH + 33 mM AMP (- - -), LDH + 33 mM NAD^+ (· · · ·), and LDH + 33 mM NADH (· · ·). The amount of protein in each run was about 200 μg. Heating rate: 10 °C/min.

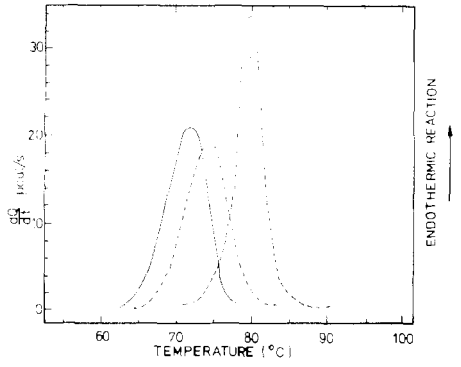

FIGURE 5: Denaturation thermograms at pH 8.1 of solutions of LDH (—), LDH + 33 mM NAD^+ (- · - ·), and LDH + 33 mM NAD^+ + 90 mM pyruvate (a ternary abortive complex) (- - -). The amount of LDH in each run was about 200 μg. Heating rate: 10 °C/min.

4B (Figures 4 and 6 and Table I). Again AMP showed practically no thermal stabilization on addition to soluble LDH, whereas NAD^+ and NADH led to an increase in T_{tr} from 72 to 74 and 79.5 °C, respectively (Figure 4 and Table I). The nucleotide concentrations were saturating (33 mM) since the K_{diss} values reported in the literature for pig heart LDH at pH around 7 are: LDH-AMP, 940 μM (Barry and O'Carra, 1975); LDH-NAD^+, 400 μM, and LDH-NADH, 0.9 μM (Stinson and Holbrook, 1973).

Figure 5 shows the thermograms obtained for soluble LDH as well as LDH in the presence of NAD^+ and NAD^+ plus pyruvate. Noteworthy is the additional increase from 74 to 79.5 °C in T_{tr} obtained after the addition of pyruvate to the LDH-NAD^+ complex. Since the pyruvate itself did not lead to any thermal stabilization of the enzyme, the increase obtained in the heat stability, identical with that due to NADH, is the result of ternary abortive complex formation (Everse et al., 1971). Besides enhancement in T_{tr} the ΔH of the transition of the ternary complex was increased by about 40% compared with the LDH-NAD^+ complex (Table I). From studies on dog fish LDH, it is known that the presence of NAD^+ leads to conformational change of the enzyme (Adams et al., 1973). The pronounced increase in stability observed for the ternary complex suggests that a further change in conformation to one thermally more stable takes place.

In Figure 6, thermograms are given of immobilized LDH obtained in the presence and absence of free NADH. When immobilized, LDH shows increased thermal stability of about 3 °C over the soluble enzyme (see Koch-Schmidt and Mosbach, 1977). Externally added NADH leads, in analogy to the

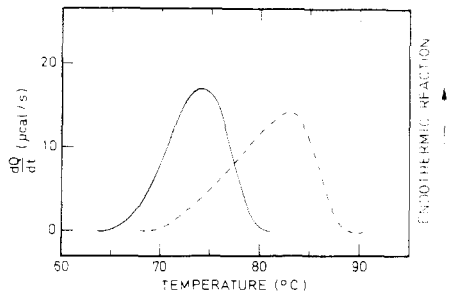

FIGURE 6: Denaturation thermograms of Sepharose-bound LDH, pH 8.1 (—), and Sepharose-bound LDH in presence of 33 mM NADH (- - -). Heating rate: 10 °C/min.

findings with soluble LDH, to an increase in heat stability of about 8 °C.

In conclusion, the technique of differential scanning calorimetry is a convenient method for characterization of a soluble, as well as an immobilized, enzyme's heat stability. It is rapid, taking only about 15 min as in the examples given here; it is highly reproducible and requires only small samples of enzyme (100-200 μg).

Obviously, this method may find more general application as a method of determining whether a change in heat stability after binding of coenzymes, coenzyme analogues, substrates, or inhibitors has occurred in both soluble and immobilized enzymes. It should also be possible to distinguish between structurally different monomers, of a dissociated oligomeric enzyme, provided they show different T_{tr} values.

In addition, the scanning procedure allows detection of minor changes and of stepwise processes possibly involved in thermal denaturation of enzymes in contrast to conventional procedures based on measurement of remaining activity, a method which cannot be utilized, for instance, for biological molecules with no catalytic activity.

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Studies on Conformation of Soluble and Immobilized Enzymes Using Differential Scanning Calorimetry. 2. Specific Activity and Thermal Stability of Enzymes Bound Weakly and Strongly to Sepharose CL 4B†

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ABSTRACT: Ribonuclease A (EC 3.1.4.22) and α-chymotrypsin (EC 3.4.21.1) have been covalently coupled, by a varying number of bonds, to Sepharose CL 4B which was activated with different amounts of CNBr. Upon increasing the number (1–8) of points of attachment between the enzyme and the matrix, the specific activities of immobilized ribonuclease A relative to its soluble counterpart decreased from 60 to 15% while the amount of protein coupled increased from 5 to 37 mg per g of sucked gel. Differential scanning calorimetry was used to determine whether the immobilization caused any changes in the physicochemical properties of the enzyme. Ribonuclease A, weakly bound to the matrix, showed almost the same behavior as the soluble enzyme. By contrast strongly immobilized enzyme exhibited a higher transition temperature (by about 5 °C) and a broader endotherm. Similar results were found for α-chymotrypsin.

Immobilized enzymes have received great attention in recent years since they represent valuable biological model systems (Srere and Mosbach, 1974). They also are of practical interest in enzyme technology (Pye and Wingard, 1974; Mosbach, 1976).

Such enzyme preparations usually have been characterized indirectly by kinetic methods and analyses such as enzyme activity determination. More direct characterization of their chemical and physicochemical properties has been neglected for the most part. Studies of this aspect have been hampered due to turbidity or opacity of such preparations and only few spectroscopic investigations have been reported to date. These include fluorometric examinations of immobilized trypsin (Gabel et al., 1971) and recent studies on Sepharose-bound proteins, such as α-lactalbumin (Barel and Prieels, 1975) and leucine aminopeptidase and carboxypeptidase A (Lasch, 1975). Preliminary reports with such preparations have appeared using circular dichroism (Zaborsky, 1974) and electron spin resonance (Reiner and Siebeneick, 1974). An important aspect of an immobilized enzyme is its conformation. Precise knowledge of this can give insights into its specific activity and thermal stability. We have therefore investigated an alternative method to spectral analysis: differential scanning calorimetry (DSC).[1] With this method an overall thermal behavior of the various enzyme preparations can be obtained easily and conclusions can be drawn as to enzyme conformation. Further information gained about the heat stability is of practical interest.

The chemical characterization of immobilized enzyme preparations has received little attention while major effort has been devoted to the nature of the support chosen. The kind of

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[1] Abbreviations used: DSC, differential scanning calorimetry; T_{tr}, transition temperature; ΔH_{tr}, enthalpy of transition; TNBS, 1-trinitrobenzenesulfonic acid; Polyox, poly(hydroxy ethylene glycol).