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Stabilization of glucose oxidase on glycation induced nano-fibrils: New insight in enzyme immobilization

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Abstract : Amyloids are interesting biomaterials with useful properties including high strength and resistance to degradation. These characteristics make amyloids suitable nano-structure candidates for bio-applications, including enzyme immobilization scaffold at nano-scale dimensions. Here, glycation induced bovine serum albumin (BSA) nano-fibrils were used as a scaffold for glucose oxidase (GOx) immobilization and the kinetic parameters optimum temperature and pH of the free and immobilized GOx were compared. The covalently bound GOx on BSA amyloid nano-fibers oxidized glucose to release hydrogen peroxide that offers a significant antimicrobial property against *E. coli* to the immobilized enzyme product. However, the enzyme's catalytic performance (k_{cat}/K_m) was decreased due to the covalent immobilization on nanofibrils. In addition, a broadening and an alkaline shift in the temperature and pH profiles of the enzyme was observed. These changes were concomitant with improved stability of the GOx upon immobilization. Together our results show that BSA nano-fibrils provide a suitable nano-structure for immobilization of GOx with enhanced stability and conserved catalytic activity.

Keywords : Amyloid; Catalytic nano-fibers; Glucose oxidase; Enzyme immobilization.

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INTRODUCTION

Materials at nano-scale dimensions have a wide variety of bio-applications, including enzyme immobilization. Proteins and peptides are self-assembled to form amyloid nanostructures[1,2]. These versatile bio-materials can be used as nano-scaffold to support biological agents such as enzymes[3]. Amyloid fibrils as filamentous ordered assemblies are formed by many proteins and peptides when subjected to glycation and denaturing conditions including extreme pH, temperature and ionic strength[4-8].

The efficiency of proteins and peptides transition to amyloids is governed by intrinsic and extrinsic factors including protein type and environmental conditions. Amyloids are composed of β strands, stabilized mainly by hydrophobic interactions and compact network of hydrogen bonds making them perpendicular to the fibril axis[9]. In protein fibrilogenesis via glycation areversible Schiff base formation is followed by Amadori rearrangements and generation of various glycated end products[10,11].

Amyloidogenesis are associated with diseases known as amyloidosis, such as Alzheimer's disease (AD). In diabetes, hyperglycemia consequences glycation of proteins *in vivo* with significant changes in protein structures and functions[12,13]. These changes buildup nano-fibrillar precipitates known as amyloid plaques within tissues resulting in physiological dysfunctions. However, more evidences are being reported on beneficial functions of some amyloids that are referred to as functional amyloids and the list of their applications are also increasing[13]. These include bio-film forming or lowering of surface tension in bacteria (curli and chaplins, respectively), involvement in adhesion of fungi (hydrophobins), protection of insect and fish eggs (chorion proteins), functioning as a bio-adhesive in algae and marine parasites[14,15], and more recently it has been reported that both primary and secondary cements (for reattachment) of the barnacle (*Balanus amphitrite*) are composed of significant amounts of nano-fibrilar amyloid matrices[13]. In addition, amyloids are biomaterials that exhibit useful properties including high strength and resistance to degradation.

Amyloid fibrils have robust mechanical properties and their tensile strength (0.6 ± 0.4 *GPa*) are comparable to metal-based fibers such as Aluminum alloy (0.483 *GPa*) and Steel, high strength alloy (0.76 *GPa*). These inherent characteristics of amyloids are mainly attributed to their hydrophobic and electrostatic forces[16]. In addition, the high accessible surface areas of their amino acid residues make amyloid structures considerably attractive for biotechnological applications. These include enzyme immobilization and their applications in nanotechnology[17,18].

The study of immobilized biocatalysts and their essential role in industrial and medical approaches is a growing area of investigation[19]. For successful improvement and application of immobilized biocatalysts, the enzyme support is generally considered as the most important component. Thus, it is imperative to develop new techniques for enzyme immobilization on cost effective carriers like amyloid nano-fibers. Enzyme immobilization could offer advantages such as conserved catalytic activity, re-usability, purity and stability of the enzyme.

In the present study we report the covalent immobilization of glucose oxidase (GOx, EC1.1.3.4) using glutaraldehyde on glycation-evolved amyloid fibrils of BSA as an enzyme immobilization nano-scaffold. GOx oxidizes the β-D-glucose to gluconic acid by using molecular oxygen as an electron acceptor and the production of hydrogen peroxide. GOx is used in many applications including glucose determination in a variety of physiological fluids, preservative in the food industry, and as an antibacterial agent[20].

EXPERIMENTAL

Bovine serum albumin (BSA) Fraction V, >95% protein was from ROCHE. The membrane filter with 0.2 μm pore size (25 mm in diameter) was from Whatmann (UK). The glucose oxidase (GOx) and peroxidase (POX) were from Sigma (St. Louis, MO, USA). The glucose and sodium azide was from Merck (Germany). Thioflavin T (ThT) and Congo red were obtained from Fluka. Uranyl acetate was from PELCO. All other materials were of analytical grade. All solutions were prepared with deionized water.

Fibril preparation

Amyloid fibrils were produced in a solution of 0.75 mM BSA in PBS (pH 7.4) in the presence of 500 mM

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glucose. The solution was incubated at 40ºC for 20 weeks in the dark. The BSA solution without any carbohydrate was used as a control. Glycated samples turned brownish yellow after 4 weeks of incubation and appeared bi-phasic over 20 weeks[11]. Following incubation, samples were pelleted by centrifugation at 13,000 ×g for 60 min. The pellets were used as nano-scaffold for enzyme immobilization after meeting all the criteria for amyloids (see below).

Fluorescence analysis

The AGE formation and protein fibrillation assessment was achieved by both intrinsic and extrinsic fluorescence strategies using Carry Eclipse spectrofluorometer with the band slit adjusted to 5 nm. Protein glycation results in production of AGEs, which intrinsically emit light at 440 nm after excitation at 342 nm[10].

Thioflavin T (ThT) as a benzothiazole dye exhibits enhanced fluorescence upon binding to amyloid fibrils, and is commonly used to assess amyloid fibrils, both *ex vivo* and *in vitro*[21]. The glycated albumin (950 µl) and control samples were incubated with 50µl of 20 µM ThT for 15 minutes at room temperature in the dark. The sample's fluorescence was measured by excitation at 450 nm and the emission recorded at 490 nm. The band slit was 5 nm and each assay was repeated three times. Congo red, a specific amyloid binding dye, was also used to assess amyloid-like fibrilogenesis. Congo red binding to amyloid structures results in hyperchromocity and the red - shift. The glycated albumin (50 µl) and control samples (0.75 mM) were incubated with Congo red solution (950 µl). The absorbance spectra were recorded at the wavelength of 400-600 nm.

Transmission electron microscopy

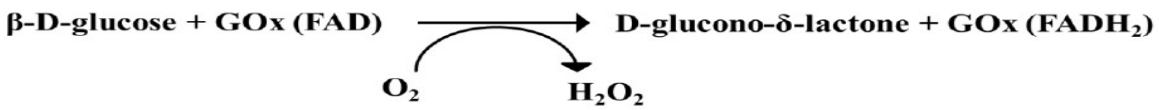
Glycation induced structures and the corresponding controls were analyzed using Transmission Electron Microscopy (TEM). In brief, samples were applied on 300-mesh grids covered with carbon coated synthetic Formvar film. After 1 minute, the buffer was wicked out using filter papers, the prepared sample was immediately stained with uranyl acetate (1% in water), and then excess solutions were wicked away and air dried[22]. Transmission electron micrographs were recorded on a ZEISS electron microscope (EM 902A) operating at 70 kV at 22000 × magnification.

Dynamic light scattering

A Zetasizer Nano S (Malvern) instrument (5 mW HeNe laser, λ=632 nm) was used for assessment of fibril hydrodynamic diameter (d_h). The glycated BSA (0.5 mg.ml^{-1}) was taken in a low volume disposable cuvette (1.5 ml volume, path length 1 cm) and hydrodynamic diameter (d_h) was evaluated several times. The results were presented in terms of distribution of d_h[23].

Enzyme assay

Protein content was estimated by Bradford method using BSA as a standard[24,25]. The colorimetric method based on Trinder's reaction was used for the determination of glucose concentration. The GOx is an oxido-reductase that catalyses the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone in the presence of oxygen while coenzyme FAD is reduced to $FADH_2$[20,26]. Hydrogen peroxide is responsible for certain antimicrobial and bactericidal effects observed in biological systems. This can be quantified using the reaction catalyzed by horseradish peroxidase (HRP) (EC 1.11.1.7) in which, hydrogen donor 4-aminoantipyrine (AAP) is stoichiometrically oxidized to yield a red quinoneimine dye with a maximum absorption at 505 nm[27].



The working solution included 80 mM K_2HPO_4, 0.1 mM EDTA, 10 mM Phenol and 5 mg.dl^{-1} 4-AAP. The NaN_3 at 0.02% was also added as an anti - fungal agent. In brief, the suspension of immobilized GOx on nano-fibers as well as the free enzyme solution, were incubated with glucose for 10 minutes, then 450 µl of working solution was added, and followed by absorbance reading at 505nm. Kinetic parameters were also determined at optimum pH and room temperature. Free and immobilized enzyme (0.8 mg.ml^{-1}) were incubated with a range of substrate concentrations (10-200 mM) and assayed for enzyme activity. Optimum pH for free

and immobilized GOx was determined by measuring the activity of free and immobilized enzyme in buffers with different pH values ranging from 3 to 7. To study the thermal stability of free and immobilized enzyme, the samples were incubated at different temperatures ranging from 5 to 80ºC, and activities were determined in time intervals[28].

Enzyme immobilization

In this study covalent cross-linking was used to immobilize GOx on BSA amyloid nano-fibers[28,29]. The optimum GOx concentration per fixed amount of the nano-scaffold was estimated upon construction of the saturation plot in which GOx at 0.1-1 $mg.ml^{-1}$ was loaded on prepared BSA amyloid nano-scaffolds. The optimum glutaraldehyde concentration was also used to minimize possible inter- and intra-molecular cross linking of GOx. In brief, 100 µl of 50 mM glutaraldehyde was added to 100 µl of amyloid fibrils, and then 100 µl of GOx was added in the concentration ranging from 0.1-1 $mg.ml^{-1}$.

The assessment of antibacterial effects

E. coli in its logarithmic growth phase was cultured on Muller-Hinton agar, which contained 10 $mg.ml^{-1}$ of glucose. Glucose free culture medium was prepared as a control. Catalytic nano-fibers were placed on blank discs, which acted as a holder. The anti-bacterial effects of the catalytic nano-fibers (GOx on amyloid fibers) were revealed after incubation at 37ºC for 24 hours[30].

RESULTS AND DISCUSSION

Glycation induced fibrillation of BSA was used to provide fibrilar nano-scaffold needed for immobilization process. The extent of fibrillation was confirmed using various methods. In the presence of glucose a single molecule of BSA is spontaneously and non-enzymatically glycated, and re-arranges to form the soluble/toxic pre-fibrils. These pre-fibrils ultimately form the non-soluble/non-toxic fibrils[31-33]. In a ''bottom-up'' strategy[3,34], glycation products orderly aggregate to form complex supra-molecular assemblies, which precipitate in the form of non-soluble fibrils in a time dependent manner[9,10]. At the early stage of the glycation, the carbonyl groups from reducing sugar side groups, and mostly lysine ε-amino groups from protein-side chains, react to form Schiff bases, which rearrange to Amadori products. These oxidized and reorganized product species are referred to as AGEs[8]. The formation of the AGE species as the function of time (weeks) is routinely confirmed by the developing of β-structures using Congo red binding, and intrinsic fluorescence ($\lambda_{ex/em}$=342/440 nm). As expected, a linear increase was observed up to 16 weeks of incubation. Here the results of benzothiazole dye, thioflavin T (ThT)-based extrinsic fluorescence assay, as the premier method of quantifying amyloid nano-fibril formation[22], is shown in Figure 1a. These binding assays further confirmed the development of a nano-fibril.

The hydrodynamic diameter of matures nano-fibrils at week 20 of incubation was determined using dynamic light scattering (DLS) at the detection angle of 632 nm relative to the incident beam (Figure 1b). The average diameter of the amyloid fibers was ~10 nm, and is consistent with previous reports[23,35]. The morphology of glycation-induced aggregates was also characterized using TEM (Figure 1c). TEM and AFM images of aggregates revealed aggregation stages from short to long fibrils and from long fibrils to light-scattering fibrils after 20 weeks of incubation.

Development of scaffolds at nano-scale is one of the fundamental issues in nanotechnology[34]. Here BSA amyloid fibrils, as bio-nano-material were used for GOx immobilization[6,36,37]. To achieve long-term stability while decreasing leakage, covalent mode of immobilization was performed. Glutaraldehyde is routinely used in two modes of applications for enzyme immobilization including carrier free (formation of intermolecular cross-linking that results in cross-linked enzymes or CLEs) and carrier binding (formation of covalent linkage between enzyme and insoluble scaffold with pendant amino groups). The reaction proceeds in aqueous buffer solution under conditions close to physiological pH, ionic strength and temperature[28,38]. Orderly aggregated nano-fibrils were used as bio-nano-scaffold in GOx immobilization to produce catalytic nano-fibers. The optimum GOx concentration for immobilization was resulted with 160 µg GOx per mg amyloid nano-fibers at 50 mM concentration of cross-linker. These were based on carrier saturation by GOx at pre-defined concentrations of nano-pre-fibers or fibers and glutaraldehyde.

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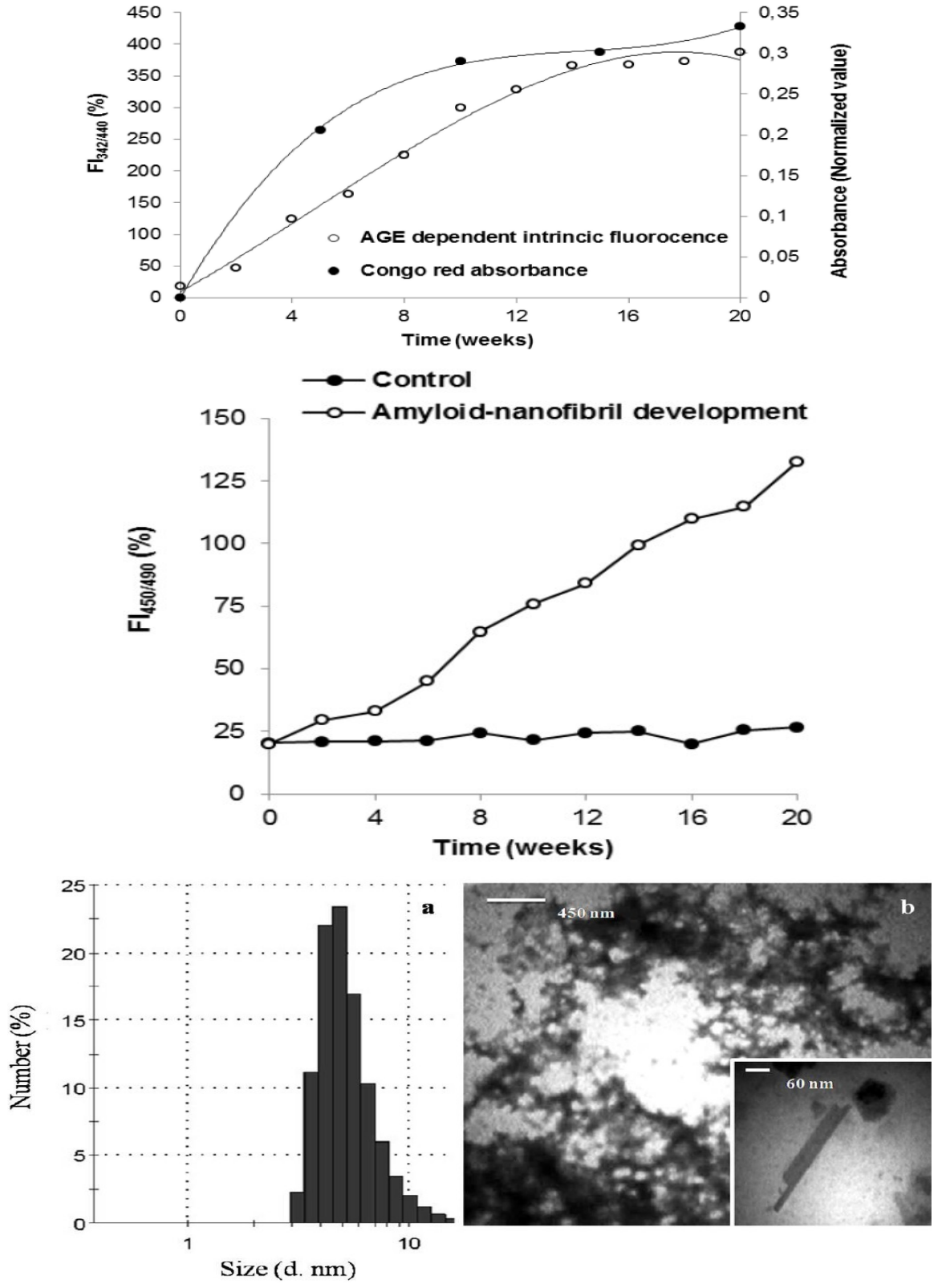

Figure 1 : ThT extrinsic fluorescence monitoring of amyloid nano-fibril formation excitation and emission maxima at 450 and 490 nm, respectively (a). BSA concentrations have been taken at 0.75 mM. DLS analysis revealed the average size of 10 nm in diameter of produced glycation–derived amyloid-fibrils. Glycated BSA after 20 weeks of incubation was diluted with distilled water and size of fibrils was evaluated on Zetasizer Nano S (Malvern) instrument (b) and TEM images present amyloid-fibrils after 20 weeks (c).

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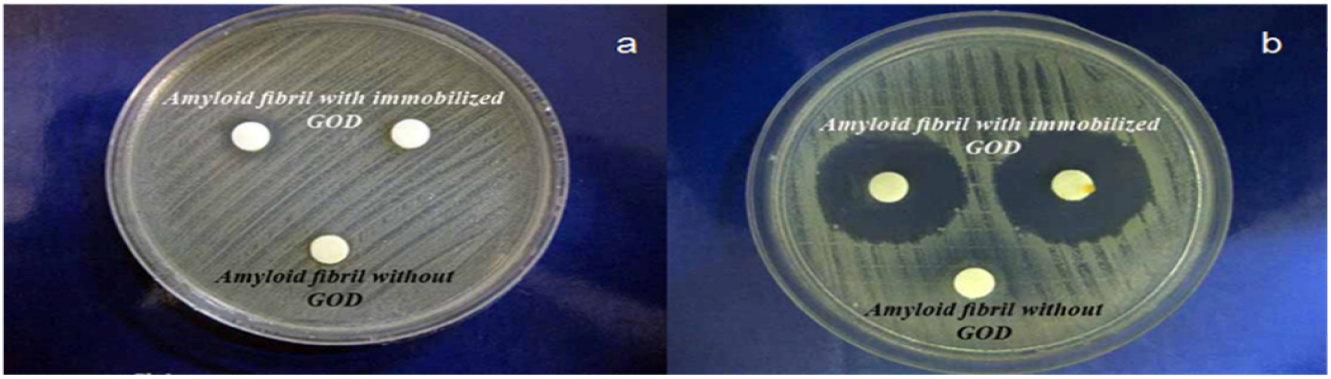

Figure 2 : Antibacterial effect of catalytic Nano-fibers against standard *E. coli* on free glucose Muller-Hinton agar (a) and glucose containing Muller-Hinton agar (b).

Due to the continual releasing of hydrogen peroxide (H_2O_2) upon oxidation of β-D-glucose by GOx, the catalytic nano-fibers represent efficient continuous antimicrobial effect at non-hazardous low concentrations of H_2O_2. To investigate the antimicrobial efficacy

TABLE 1 : Kinetic parameters of the free and immobilized GOx on glycation-derived BSA amyloid nano-fibers.

GOx	K_m (mM)	V_{max} (dA.min^{-1})	k_{cat}/K_m
Free	22.86	0.28	12.20
Immobilized	27.42	0.14	6.09

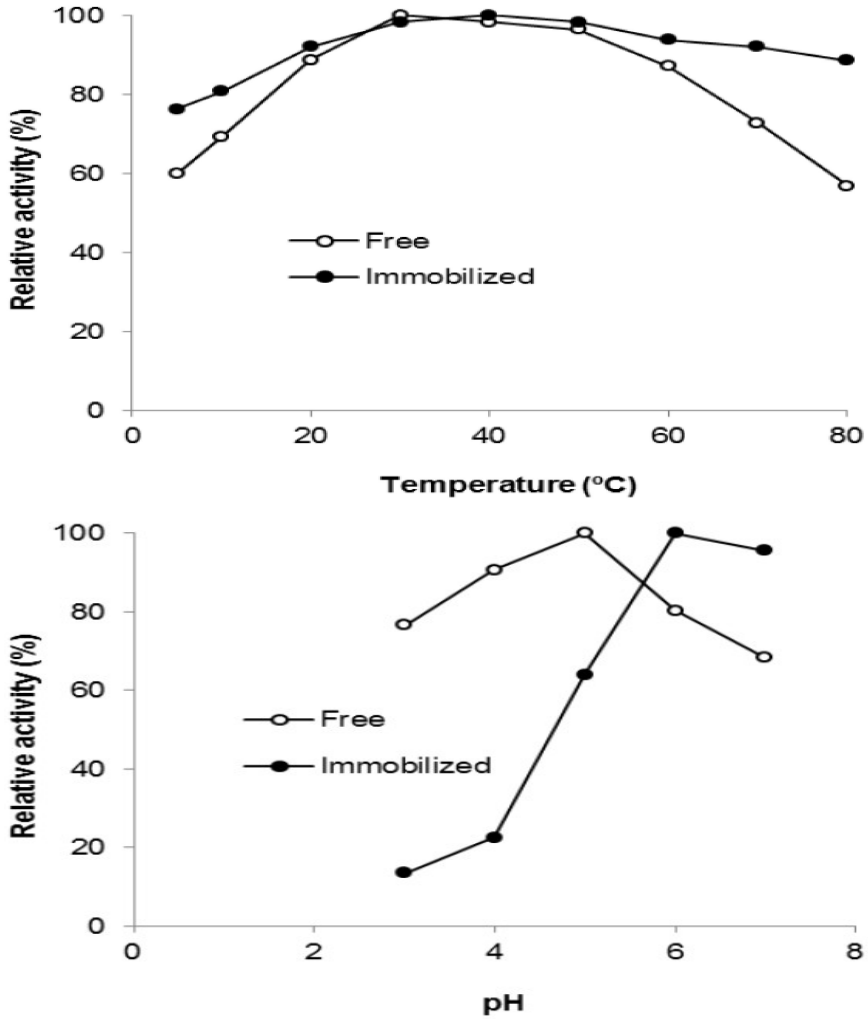

Figure 3 : Temperature (a) and pH (b) profiles of the free and immobilized GOxs on glycation-derived BSA amyloid nano-fibers.

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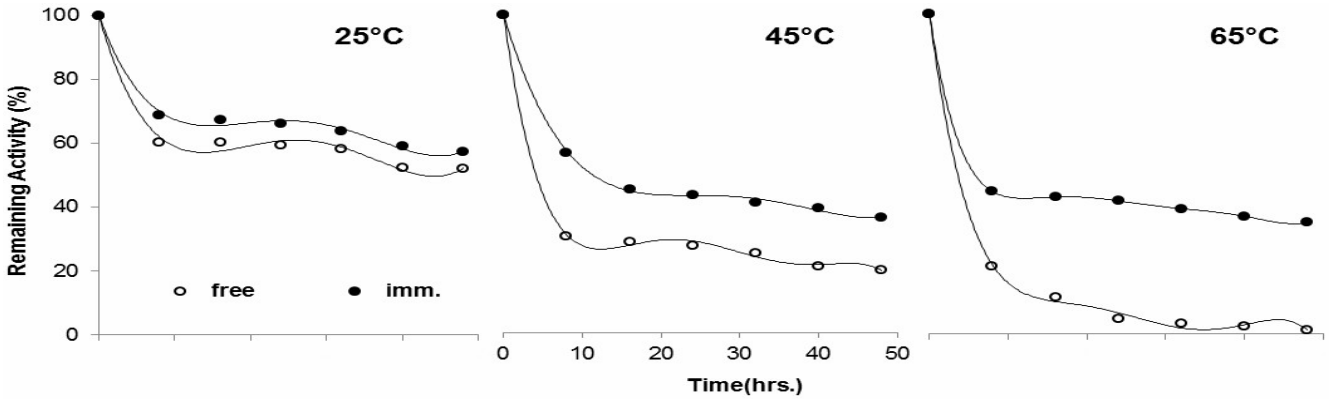

Figure 4 : Thermo-stability of free and immobilized GOx on BSA glycation–derived nano-fibrils in which remaining activities were assayed at time intervals after leaving samples at 25, 45 and 65 ºC

of the immobilization product, catalytic nano-fibers were applied to blank discs (as holder of catalytic fibers) and were placed on Muller-Hinton agar in the absence or presence of glucose (Figure 2). Covalently bound GOx molecules on amyloid nano-fibers revealed significant antimicrobial properties against *E. coli*[30].

The kinetic parameters of the free and immobilized GOx on amyloid nano-fibers were also determined. We observed an increase in the K_m and a decrease in the catalytic performance (k_{cat}/K_m) of the enzyme upon immobilization (TABLE 1). However, the catalytic activity remained sufficient to fulfill application demands, such as antimicrobial activity. These changes were attributed, at least in part, to the enhanced enzyme rigidity, a common drawback of the covalent immobilization strategy, and increased mass transfer limitations. Similar observations have been reported when GOx was immobilized on the silk fibroin membrane or on cellulose acetate–polymethyl methacrylate (CA–PMMA) membrane. In the latter case, a 1.19-fold increase in K_m value was observed, whereas V_{max} of the immobilized preparation was lower than that of free GOx.

Figure 3 shows the temperature and pH profiles for both the free and immobilized GOxs. The immobilized enzyme had a rather broad temperature profile with apparent optimum temperature at 40°C, same as the free enzyme. This represents an improvement in the temperature dependence of the immobilized enzyme activity due to the extra covalent linkages in the immobilized form (Figure 3a). The optimum pH of the immobilized enzyme was shifted to the alkaline range (pH 6) compared with the free enzyme (pH 5) (Figure 3b)[39]. This effect may reflect the change in the enzyme's active site microenvironment brought about by neighboring charged groups. It has been previously demonstrated that depending on the charge properties of the matrix, the optimum pH may undergo significant shift. The optimum pH for an enzyme bound to a negatively charged carrier shift to higher values, while immobilization on a cationic matrix has an opposite effect. Thus, the observed alkaline shift in the optimum pH for catalytic activity, as an effect of immobilization, is thought to describe the anionic nature of the amyloid nano-fibers as the matrix for immobilization. Thus, the optimum pH of immobilized enzyme mirrors the microenvironment where the enzyme is immobilized.

Experiments related to thermo-stability of the free and immobilized GOx on amyloid nano-fibers were also performed. The immobilization of GOx resulted in its improved stability (Figure 4). The immobilized GOx retained its activity after incubation at temperatures of 25, 45 and 65ºC, and thus, it is more efficient than the free enzyme. However, the preference of the immobilized enzyme was at a higher temperature.

ABBREVIATIONS

4-AAP	:	4-aminoantipyrine
AGE	:	advanced glycated end product
BSA	:	bovine serum albumin
DLS	:	dynamic light scattering
GOx	:	glucose oxidase
TEM	:	transmission electron microscopy
ThT	:	thioflavin T

CONCLUSION

The beneficial function of ordered protein aggregates as amyloids have been recognized in numerous studies. Amyloids are interesting nano-material with high strength and stability at extremes of pH, temperature or pressure and they are also resistant to proteolysis and dehydration. In this study we report glycation induced bovine serum albumin (BSA) amyloids as a nano-scaffold for glucose oxidase immobilization to produce catalytic nano-fibers with anti-microbial properties. This immobilization strategy follows an increase in storage stability of GOx and a broadening/alkaline shift in optimum temperature and pH.

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