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Short communication

Peculiarities of a novel bioenzymatic reactor using carbon nanotubes as enzyme activity enhancers: Application to arginase

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ABSTRACT

Multiwalled carbon nanotubes have been entrapped in a porous monolithic chromatographic support. This support was used for the covalent immobilization of the arginase enzyme a novel target in hypertension. The effect of the nanotube (NT) amount into the monolith was analyzed. The obtained results demonstrated the ability of carbon nanotubes to increase significantly the performance of this novel bioactive support.

1. Introduction

Arginase is an enzyme converting L-arginine to urea and L-ornithine. The two isoforms of this enzyme are expressed by endothelial and vascular smooth muscle cells (VSMC). Recently Guillaume's group demonstrated that arginase inhibitors prevented the development of hypertension and improved aortic endothelial function [1–3]. Arginase inhibition could thus represent a novel strategy in hypertension. Thus it was necessary to focus on the development of arginase bioenzymactic reactor to study arginase inhibitors. Studies from our laboratory reported the development of bioactive supports based on the covalent binding of arginase to a solid carrier material [4,5]. Multiwall carbon nanotubes (MWCNTs) [6–9], exhibit physical properties that make them potentially useful in the development of chromatographic supports both in high performance liquid chromatography (HPLC) and gas chromatography GC [10–15]. No studies were reported to analyze the interest of carbon nanotubes in the development of bioreactors using a porous monolithic support. This article demonstrated the use of carbon nanotubes entrapped in a polymeric chromatographic support to enhance the performance of the enzymactic reactor

2. Experimental

2.1. Equipment

The HPLC system consisted of a Waters HPLC pump 501 (Saint-Quentin, Yvelines, France), an Interchim rheodyne injection valve, Model 7125 (Interchim, Montluçon, France), fitted with a reverse 20 (L sample loop, a Merck L4000 variable-wavelength UV spectrophotometer detector, and a Merck D2500 chromato-integrator (Nogent sur Marne, France). The chromatographic column tube (20 mm × 4.6 mm) was obtained from Interchim (Montluçon, France). Specific surface area and porosimetry of the chromatographic supports were determined by the Micromeritics' ASAP 2020 Accelerated Surface Area and Porosimetry Analyser (Micromeritics France SA, Verneuil en Halatte).

2.2. Reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), azobisisobutyronitrile (AIBN), 1-dodecanol, cyclohexanol, ammonium hydroxyde, glutaraldehyde, sodium cyanoborohydride, monoethanolamine, m-nitro-aniline (m-NA) and arginase were obtained from Sigma–Aldrich (Paris, France). N^{ω}-hydroxy-L-arginine (NOHA), N^{ω}-hydroxy-nor-arginine (nor-NOHA) and (S)-(2-boronoethyl)-L-cysteine (BEC) were obtained from Bachem (Germany). All chemicals were used as received with the exception of EDMA and GMA, which were redistilled. Water was obtained

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from an Elgastat water purification system (Odil, Talant, France), fitted with a reverse osmosis cartridge. Pristine multiwalled carbon nanotubes (5–10 nm i.d., 10–30 nm o.d., 1–2 μm length, batch SN2303) were purchased from Aldrich Co. (Paris, France) and were used as received. All the other chemical products were of analytical grade and all the buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for HPLC. The arginase substrate, 1-nitro-3-guanidinobenzene (NGB), was synthesized as explained in a previous paper [16].

3. Method

3.1. Entrapment of carbon nanotubes (NTs) in the chromatographic support

The monolithic stationary phase was directly prepared by in situ thermal-initiated polymerisation within the chromatographic column tube. Glycidyl methacrylate (28% (m/m)), ethylene dimethacrylate (12% (m/m)) and a small specific amount of NT × (% (m/m)), were added in the appropriate porogenic agents 1-dodecanol (6% (m/m)) and cyclohexanol (53.2% (m/m)). After mixing a homogeneous black solution was obtained due to the dispersion of NT. Then, azobisisobutyronitrile (0.8% (m/m)) with respect to monomers) was added to the mixture. The solution was then sonicated for 30 min, purged with nitrogen gas for 15 min at 25 °C to remove oxygen. The ends of the stainless-steel tube were connected with plastic tubes and then sealed at the bottom, filled with the above polymerization mixture and then sealed at the top. Subsequently, the polymerisation was performed in a water bath at 55 °C for 24 h. After the polymerization, the seals and plastic tubes were removed. The resulted column was provided with fittings, and connected to an HPLC pump. Ammonium hydroxide was thus pumped through the column at a flow rate of 0.4 mL/min until the effluent was basic to provide primary amine functionalities due to the reaction of ammonium hydroxide with epoxy groups of the poly(glycidyl methacrylate-co-ethylene dimethacrylate). The NH2-column was thus flushed with water until the effluent had neutral pH.

3.2. Arginase attachment to the pore surface of the NT chromatographic support

The immobilization of arginase via the amino groups of the enzyme on the NH_2-column activated with glutaraldehyde, a well known amine-reactive cross linker [17,18], was carried out as follows. The column was first washed for 1 h with a mobile phase consisting of phosphate buffer (50 mM, pH 7.00) at 0.4 mL/min. Then the NH_2-capillary was activated by recycling a solution containing 13% glutaraldehyde in phosphate buffer (50 mM, pH 6.00) for 12 h. After that the column was flushed with phosphate buffer (50 mM, pH 6.0). An aliquot of 800 μL of arginase solution in phosphate buffer (50 mM, pH 8.5) was recirculated through the column at a flow rate of 0.4 mL/min for 16 h, flushing and back flushing every 15 min during the first hour, every 30 min during the following 3 h. After immobilization, the UV absorbance decrease of the arginase solution was determined to calculate the unreacted enzyme quantity. The arginase column was thus flushed with a 10 mL solution of cyanoborohydride (0.1 M) in phosphate buffer (50 mM, pH 6.00) for 5 h at 25 °C to reduce the Schiff bases. After that, the column was flushed with a phosphate buffer (50 mM; pH 6.00) to remove the unreacted reagent, and flushed with a solution of monoethanolamine (0.2 M) in phosphate buffer (50 mM, pH 8.50) at room temperature. Finally, the arginase column was washed with phosphate buffer (50 mM, pH 7.4) for 1 h at a flow-rate of 0.4 mL/min. When not in use, the arginase-column was stored at 4 °C in phosphate buffer (50 mM, pH 7.4) containing 0.1% sodium azide.

3.3. Determination of the arginase activity, kinetic parameters and chromatographic data

The determination of the activity of the immobilized enzyme was explained in [5] and was expressed in terms of active units (U) using the chromophore m-nitro-aniline (m-NA) from the reaction between the enzyme with the substrate 1-nitro-3-guanidinobenzene yielding products urea plus m-NA. Briefly, the arginase stationary phase was conditioned with the mobile phase i.e., (0.1 mM Tris–HCl) buffer pH 7.4 containing 10 mM $MnCl_2$ since arginase required Mn^{2+} before it can become activated. Flow-rate was set at 0.4 mL/min and UV detection at 372 nm corresponding to the maximal absorption of m-NA (at this wavelength, the absorption of NGB is much less than of m-NA [16]). Aliquots of 20 (L of NGB prepared in the (0.1 mM Tris–HCl) buffer pH 7.4 were injected at increasing concentration (range comprised between 3 and 300 mM) and the Michaelis–Menten trend was found by plotting the rate of enzymatic reaction (V) against the substrate concentration [S]. The kinetic parameters V_{max} and K_m were obtained thanks to the Lineweaver Burk plot as explained in [16]. The retention of the m-nitro-aniline (m-NA) on the NT-chromatographic support was evaluated using the retention factor $k = (t - t_0)/t_0$, where t was the retention time of m-NA on the chromatogram and t_0 the column dead time determined using the mobile phase peak. The efficiency of the column reflecting the band broadening was characterized by estimating the height equivalent to a theoretical plate $h = L/(5.54(t/\delta)^2)$ where L was the column length and δ was the peak width at half-height.

4. Results and discussion

The white-colored monolithic supported with no NTs prepared at 55 °C exhibit surface area of 39 m^2/g. The pore size was 1.70 μm. After treatment with ammonium hydroxide, X-ray photoelectron spectroscopy showed that this monolith contains 0.98 atomic% of nitrogen confirming the presence of primary amine functionalities on the pore surface. The UV absorbance decrease the arginase solution before and after the enzyme immobilisation procedure on the chromatographic support described in Section 3.2 was determined to calculate the unreacted enzyme quantity. The immobilization yield of the arginase enzyme on the pore surface of the polymer was thus found to be 13.4%. Addition of NT to the polymerisation mixture affords grey colored with no change in the surface area, pore size and immobilization yield for 0.10% or 0.30% of NT. This finding is corroborated with negligible change in the back pressure of the column at all tested flow rates (±3%). With the following chromatographic conditions i.e., mobile phase 0.1 mM Tris–HCl buffer pH 7.4, 10 mM $MnCl_2$, flow rate = 0.4 mL/min, column temperature = 25 °C and detection wavelength = 372 nm, the chromatographic peak of m-NA obtained on the arginase column without NT was given in Fig. 1A. The corresponding retention factor (k) and column efficiency (h) were respectively equal to 1.32 and 12.6 μm. To confirm the chromatographic peak on Fig. 1A and B was the m-NA peak, a standard compound was injected in similar chromatographic conditions and the retention factor obtained was the same. Entrapment of NT into the polymer structure results an increase in both retention and efficiency. A column efficiency of 7.4 μm determined at a flow rate of 0.4 mL/min was achieved at a content of 0.30% NT with a retention factor equal to 2.55 (Fig. 1B). A sharp and symmetrical peak was obtained for m-NA in its isocratic mode elution. To confirm these results, four other arginase stationary phases were developed where the amount of NT in the

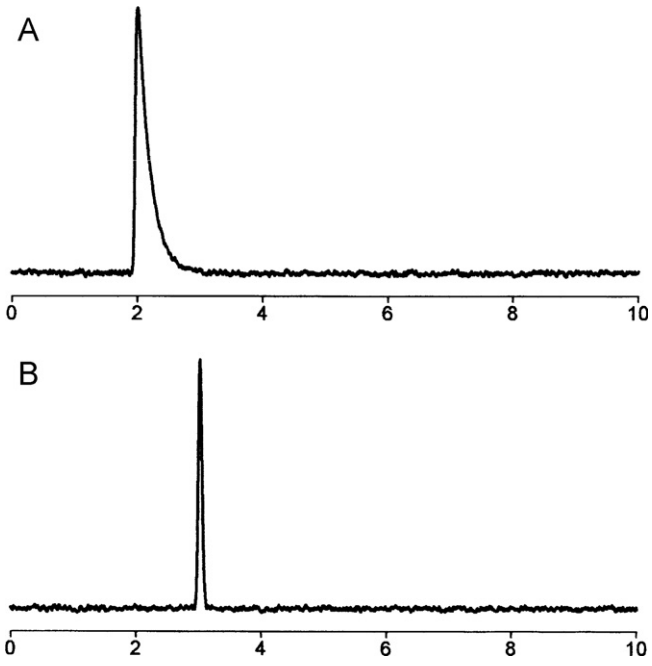

Fig. 1. Chromatogram obtained after the injection of 10 μL of 200 mM NGB for (A) $x = 0\%$ and (B) $x = 0.30\%$ of NT in the monolithic chromatographic support. Mobile phase: 0.1 mM Tris–HCl buffer pH 7.4, 10 mM $MnCl_2$. Flow rate: 0.4 mL/min. Column temperature: 25 °C. Detection wavelength: 372 nm (corresponding to the peak of m-NA).

polymerisation mixture was equal to 0.1%; 0.15%; 0.20%; 0.25%. All the columns were repeated three times. Beyond 0.30%, NT dispersed with great difficulties in the polymerisation mixture and the obtained polymer tends to crack. Figs. 2 and 3 confirmed that increasing amount of NT in the polymerisation mixture enhanced both retention and column efficiency. Andre et al. compared carbon nanotubes to graphite sheet (sp^2) carbon roles in tube [19]. The π–π interactions between the aromatic group of m-NA with the surface of nanotubes are thought be responsible for high retention. Fig. 4 reports all the data acquired on the evolution of the relative arginase activity (RAA) (i.e., arginase activity relative to the value at $x = 0$) when the NT amount x increased from 0% to 0.30%. Looking at the experimental data, it is evident that the RAA increased when x increased. These results demonstrated clearly the interest of the entrapment of carbon nanotubes in the polymer for arginase activation. NT allowed the adsorption increase of the freshly generated product (m-NA) on the arginase monolithic support thus enhanced the equilibrium displacement towards the product formation in

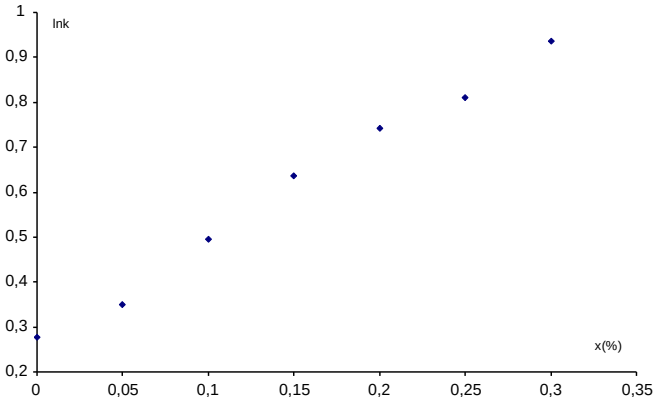

Fig. 2. ln k values versus the percentage of NTs into the chromatographic support x (%). The experimental chromatographic conditions were the same as in Fig. 1.

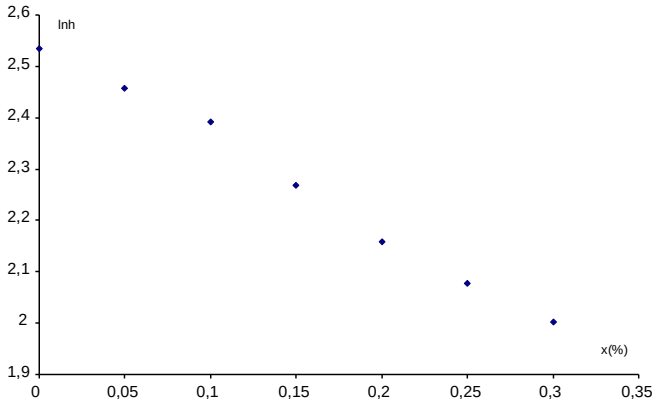

Fig. 3. ln h values versus the percentage of NTs into the chromatographic support x (%). The experimental chromatographic conditions were the same as in Fig. 1.

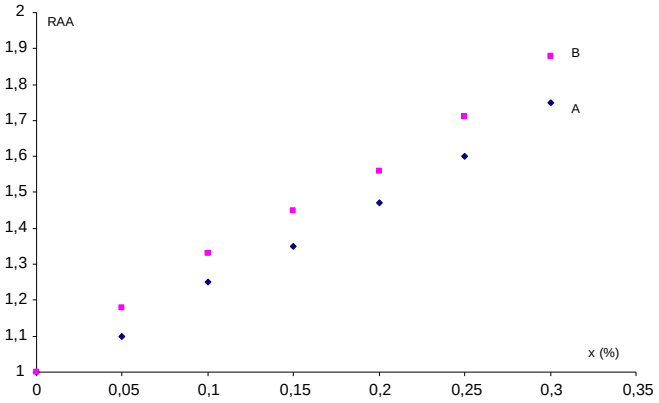

Fig. 4. Relative Arginase Activity (RAA) (i.e., arginase activity relative to the value at $x = 0$) vs x(%): (A) pore size 1.70 μm (B) pore size 0.49 μm. The experimental chromatographic conditions were the same as in Fig. 1.

the mobile phase. NT helped to increase product formation by 75% when the NT amount in the polymer was 0.30%. Concerning the kinetic parameters, Table 1 indicated that the K_m values did not change significantly with x and were similar as those obtained in a previous paper using the same mobile phase buffer [5]. This result can be explained by the fact there was no change on the binding site of the enzyme structure, so the enzyme affinity towards the substrate did not change either. Whereas the V_{max} values with x were increased (Table 1). These data indicated classical activation kinetic without a significant influence on the rate of substrate binding [20] and suggested that NTs affect positively proximity and enzyme orientation for its interaction with the substrate NGB. With a polymerisation temperature of 70 °C, the pore size of the monolithic support decreased and was 0.49 μm. Fig. 4 demonstrated that

Table 1
Effect of the amount NT on K_m (mM) and V_{max} ((mol/min) for arginase catalyzed NGB hydrolysis after 200 mM NGB injection.

x (%)	K_m (mM)	V_{max} ((mol/min)
0.00	13.7 (0.1)	142.2 (0.3)
0.05	13.9 (0.2)	149.3 (0.3)
0.10	14.1 (0.4)	153.4 (0.4)
0.15	13.8 (0.3)	161.7 (0.5)
0.20	14.0 (0.1)	169.7(0.5)
0.25	13.9(0.2)	176.8(0.3)
0.30	14.2(0.3)	183.5(0.4)

Mobile phase: 0.1 mM Tris–HCl buffer pH 7.4, 10 mM $MnCl_2$, flow-rate: 0.4 mL/min, IMER temperature: 25 °C, detection wavelength: 372 nm, standard deviations were in parentheses.

decreasing the pore size of the monolithic support increases the relative arginase activity. As the pore size decreased, the surface area of the stationary phase increased which allow achieving an optimal enzyme density on the surface of the polymer. As well, if r_p was the average reaction rate for the whole pore and r_s was the reaction rate on the pore surface, the effectiveness factor η can be introduced to study the effect of the pore size on the arginase activity. η was equal to r_p/r_s and the effectiveness factor must be close to 1 for an optimal arginase activity. In the case of a Michaelis–Menten kinetics, $\eta = 1/\emptyset(1/\tan h(3\emptyset) - 1/(3\emptyset))$ where $\emptyset$ was the Thiele modulus [21,22]. For spherical pores $\emptyset = \kappa R$ where R was the pore radius and κ was a term which depended on the Michaelis–Menten constants of the enzyme. The effectiveness factor η is close to 1 when the Thiele modulus is close to 0. Therefore it is beneficial to have pores of a small radius. However, such small pores lead to a more pronounced column back pressure which soon will become a limiting parameter. To confirm the use of this enzymatic reactor for the research of inhibitors, arginase inhibition by three well-known inhibitors i.e., NOHA, nor-NOHA and BEC was analyzed using this NT arginase column. The determination of inhibitory potency (IC50) of these three inhibitors was determined as explained in a previous paper [5]. Briefly, the inhibition curves were obtained by injecting simultaneously both the substrate at a fixed saturating concentration and inhibitors at increasing concentration. Increasing reduction of the m-NA peak area on the chromatogram when compared to the area obtained by the sole substrate was observed for increasing inhibitor concentration. For $x = 0.30\%$ and a polymerisation temperature equal to 55 °C with a 0.1 mM Tris–HCl buffer pH 7.4, 10 mM $MnCl_2$ as mobile phase, the IC50 values were equal to 11.0 (mol/L for NOHA, 0.56 (mol/L for nor-NOHA and 0.52 (mol/L for BEC. The values obtained were similar as those obtained for the biological substrate arginine [23].

5. Conclusion

For the first time, the direct implication of the entrapment of carbon nanotubes into a monolithic support to enhance the performance of an enzymatic reactor was demonstrated. This enhancement increased with the amount of NT into the polymerisation mixture. This novel arginase monolithic support exhibited as well excellent chromatographic retention and efficiency for the generated product from the enzymatic reaction. This novel arginase reactor could be a useful tool for the research of novel arginase inhibitors.

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