JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 9, No. 3, March 2007, p. 686 - 689

Adsorption behavior of hyaluronidase onto silver nanoparticles and PMMA bone substitute

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Surface enhanced Raman scattering (SERS) and ATR-FTIR spectroscopy was applied in this work, in order to get information about the adsorption behavior of the title macromolecule with respect to different surfaces. In this case, the silver substrate can be considered as artificial substrate and the investigations regarding the mechanisms of adsorption can be useful in order to elucidate the active site properties of this enzyme. Our purpose is to study the adsorption mechanism of hyaluronidase onto silver nanoparticles and PMMA (polymethyl methacrylate) substrates as well as qualitative and quantitative aspects regarding perturbations of protein secondary structure (α-helix, β-sheet and unordered structures) upon adsorption, using deconvolution techniques.

(Received November 15, 2006; accepted December 21, 2006)

Keywords: SERS, Raman, FTIR, Hyaluronidase adsorption, Conformational change

1. Introduction

Hyaluronidase is a glycoprotein containing 5% mannose and 2.17% glucosamine, composed by four subunits of 14000 Da each and a total molecular weight of 55000 Da. This enzyme hydrolyzes the endo-N-acetylhexosaminic bonds of hyaluronic acid (HA) and chondroitin sulfuric acids A and C (but not B), primarily to tetrasaccharide residues [1]. Recently [2] it has been demonstrated the possibility that elevated levels of HA/HAase could be a marker for prostate cancer progression based on their understanding that many tumors share similar characteristics of growth and metastasis. By analogy with other enzymes that hydrolyze polysaccharides, certain assumptions could be made, such as that the two acidic amino acids, mostly two glutamic acid residues are part of the active site. In addition, it has been shown that the region where the polysaccharide binds to some of these enzymes is correlated with the aromatic side chain of several tryptophan and tyrosine residues [3]. FTIR and FT Raman spectroscopy techniques have the potential to answer many of the questions pertaining to the chemical detail of enzyme mechanisms and adsorption to biomaterials. However, such application has been held back by the twin difficulties of lack of sensitivity and selectivity for large macromolecular complexes. Overcoming these difficulties, surface enhanced Raman scattering (SERS) was applied in this work, in order to get information about the adsorption behavior of the title macromolecule. In this case, the silver substrate can be considered as artificial substrate and the investigations regarding the mechanisms of adsorption can be useful in order to elucidate the active site properties of this protein.

As the hyaluronic acid is an important constituent of extracelular matrix of vertebrates, the adsorption mechanism to implants materials is an essential aspect of the cascade of biological reactions taking place at the interface between synthetic material and biological environment. The type and amounts of adsorbed proteins mediate subsequent adhesion, proliferation and differentiation of cells as well as depositing of mineral phase. Polymethyl methacrylate (PMMA) based biomaterials are extensively used in the past three decades as bone substitutes and teeth due to their high biocompatibility, bioactivity and mechanical properties, ensured from the clinical experience [4-6].

2. Experimental

Hyaluronidase from bovine testes, type VIII, (EC 3.2.1.35), lyophilized powder, aprox.300 units/mg (molecular weight 55 kDa- four subunits of 14 kDa each) from Sigma Aldricht, has been used without further purification. The stock solution of enzyme was prepared at 5 mg/ml in 0.1 M sodium phosphate buffer pH 5.3 with 0.15 M sodium chloride. Immediately prior to use it was diluted further in the same buffer. The sodium citrate-reduced Ag colloid has been obtained using the classical procedure Lee-Meisel [7]. Small amount of protein solution was dropped in 3 ml Ag colloid, the protein concentration for SERS measurements being 10^{-4} mol/l and 5.10^{-5}mol/l, respectively. PMMA (polymethyl methacrylate) was purchased from Stryker Howmedica Osteonics, commercially available as BIOLOS3®. After polymerization, small plates of PMMA were incubated for 24 hours at 37 °C in protein solution containing 2 mg/ml in 0.1 M sodium phosphate buffer pH 5.3 with 0.15 M sodium chloride. After drying process, the surfaces were

analyzed by ATR FTIR spectroscopy. FT-IR spectra of the powder hyaluronidase have been recorded using a FT-IR Equinox 55 Bruker spectrometer with an attenuated total reflectance (ATR) module, in the region 4000-800 cm^{-1}, with a scanning speed of 32 cm^{-1} min^{-1} and the spectral width 2.0 cm^{-1}. Curve fitting was performed by setting the number of component bands found by second-derivative analysis with fixed bandwidth (12 cm^{-1}) and Gaussian profile. The best-average fit gave the intensity of each component band for each spectrum. The area under each peak was used to calculate the percentage of each component and finally used to analyze the percentage of secondary structure components. An integrated FRA-106 S Raman module was employed for recording the FT-Raman spectra, using an Nd:YAG laser operating at 1064 nm line for excitation. The laser power was 350 mW and 200 scans were collected for each spectrum. The detection of the Raman signal was carried out with nitrogen cooled Ge detector. The spectral resolution was 4 cm^{-1}.

SERS spectra of enzyme aqueous solution in Ag colloid were obtained using a Dylor LabRam micro-Raman spectrometer with a 10x microscope objective. An argon –ion laser operating at 514.5 nm with an output power of 250 mW was employed for excitation. A number of 10 cycles x 20 scans were accumulated. The detection of the Raman signal has been performed with a Peltier cooled CCD detector.

3. Results and discussion

The ATR- FT-IR spectrum of hyaluronidase (lyophilized powder) is presented in Fig. 1 reviling the conformationally-sensitive amide bands and the comparison between the FT Raman and SERS spectrum on silver nano-substrate is presented in Fig. 2. The tentative assignments of the compared vibrational modes are listed in Table 1, according to the related references [8,9]

Amide I vibrations represents the in plane C=O stretching, weakly coupled with C-N stretching and CCN deformation. In our ATR FTIR spectrum (fig.1) is represented by the very strong band at 1654 cm^{-1} while the corresponding FT Raman is shifted to lower wavenumber 1660 cm^{-1} (Fig. 2) . Amide II vibrations derives mainly from in plane N-H bending (40-60%), the C-N (18-40%) and C-C (10%) stretching vibrations. The very strong band at 1535 cm^{-1} in the ATR FTIR spectrum has a weak correspondent at 1553 cm^{-1} in FT-Raman. In the SERS spectrum, both amide I and amide II contributions are represented through the broad and strong band ranging from 1595 to 1550 cm^{-1}. Amide III is a more complex vibrational mode representing in phase combination of N-H in plane bending and C-N stretching with contributions from CC stretching and CO in plane bending, depending on the details of the force field. In Fig. 1, this contribution is represented as a weak band at 1308 cm^{-1}, the corresponding FT Raman is a broad band of medium intensity ranging from 1337 to 1317 cm^{-1} and the SERS corresponding is a weak to medium band at 1326 cm^{-1}. The stretching vibrations C-O-C, C-O and C-O-H are selectively enhanced in the SERS spectra, emphasized by the strong band at 1159 cm^{-1}. The strong intensity of the band at 219 cm^{-1} observed in the SERS spectra allowed the presumption of oxygen-adsorption from the extremal oxygen-containing functional groups from the complex molecular structure.

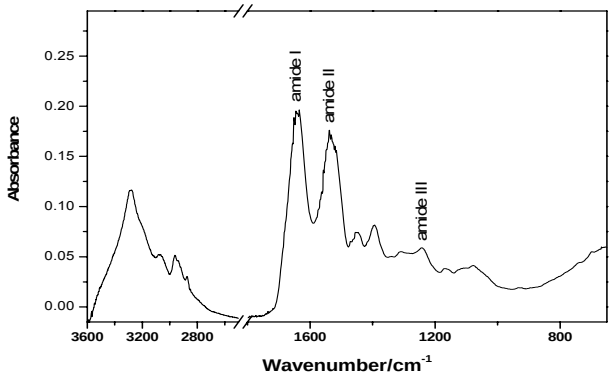


Fig.1. ATR- FT-IR spectrum of hyaluronidase (lyophilized powder).

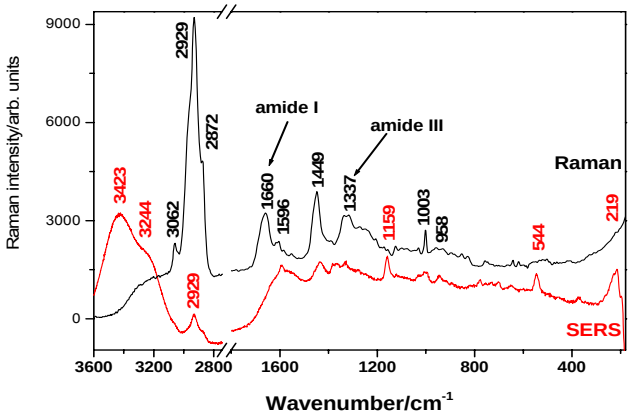


Fig. 2. Comparison between FT-Raman and SERS spectra of hyaluronidase. Large differences can be observed both in bands position and relative intensity. Experimental parameters: SERS excitation 514.5 nm, power 250 mW; Raman excitation 1064 nm, power 350 mW.

Table 1. Vibrational ATR FTIR, FT Raman and SERS data (cm^{-1}) of hyaluronidase and their proposed assignments.

ATR-FTIR (cm^{-1})	FT-RAMAN (cm^{-1})	SERS (cm^{-1})	Vibrational Assignment
	-	3423	N-H stretch
3283 *vs*	-	*vs*	O-H stretch (aqueous
3066 *m*	3062	3244	colloidal Ag)
2960 *m*	*w*	*sh*	C-H stretch
2871 *w*	2929	-	
1654 *vs*	*vs*	2929	Amide I, C=C
-	2872 *sh*	*m*	
1535 *vs*	1660 *vs*	2872	Amide II
1452 *m*	1597	*sh*	CH_2, CH_3 bend, C-O-H
1391 *s*	*w*	1633 sh	bend.
1308 *w*	1553	1595 *vs*	C-H bend
1163 *w*	*w*	1550	Amide III
1073 *w*	1449 *vs*	*sh*	
	1382 *w*	1435 *vs*	C-O-C, C-O, C-O-H
	1337 *m*	1371 *w*	stretch
	-	1326 *w*	C-O-C stretch, O-H
		1159	bend, C=O bend
	1003	vs	C-C-O stretch
	m	1031 sh	Ag-O
	958 *w*	998 m	
		947 w	
		544 m	
		219 s	

The ATR-FTIR spectra before and after incubation of small plates of PMMA in hyaluronidase buffer solution (see experimental section) are presented in Fig. 3. The reference spectrum in Fig. 3a indicate the details of functional groups in polymethyl methacrylate; a sharp and intense band at 1726 $cm^{-1}$is due to the presence of ester carbonyl group stretching mode, a broad band at 1438 cm^{-1} due to C-H bending and the peaks in the range 1260-900 cm^{-1} are assigned to O-C-O, C-CH_3 stretching and C-COO vibrations . The adsorption of hyaluronidase to PMMA surface after incubation is emphasized in Fig. 3b by the amide I band at 1646 cm^{-1} and amide II at 1545 cm^{1}.

According to literature [10,11] the shift of amide I to lower numbers after adsorption, indicates a modification of the secondary structure, while a decreased intensity of the amide II band suggests an increased accessibility of amide bond to water, which are clearly emphasized in our spectra (Fig. 1 and Fig. 3b). Fourier deconvolution of amide I, II and III as well as band fitting procedure were performed in order to obtain more qualitative and quantitative information related to the adsorption induced conformational transitions [12,13]. Fig. 4 shows the deconvolution of amide I band of the native (a) and adsorbed enzyme (b) on PMMA surface. The five components of amide I native enzyme are related to the α helix structure (1648 cm^{-1}), β sheet (1631 cm^{-1}), β – turns (1661 cm^{-1}), aggregates (1619 cm^{-1}) and random coil (1685 cm^{-1}). The related components of the adsorbed enzyme are shifted toward higher wavenumbers and the percentage area of each component is drastically changed. The α helix content decreased from 32.64% to 27.68% upon adsorption; β sheet decrease also from 36.15% to 18.8% accompanied by an increase of β- turns structure from 9.1% to 20.8 %, the aggregates from 7% to 11.4% and random coil from 14% to 21.8 %.

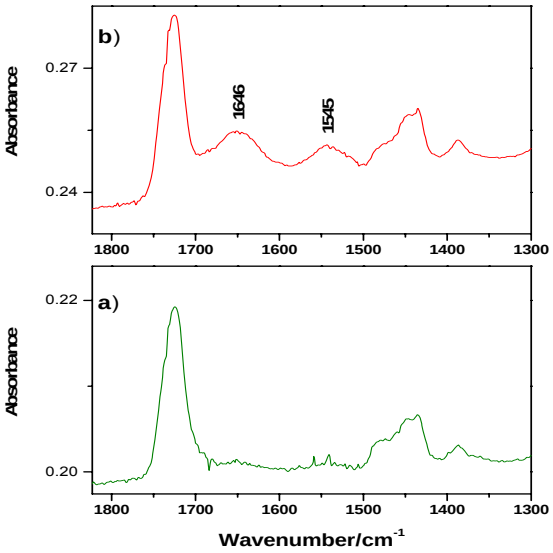
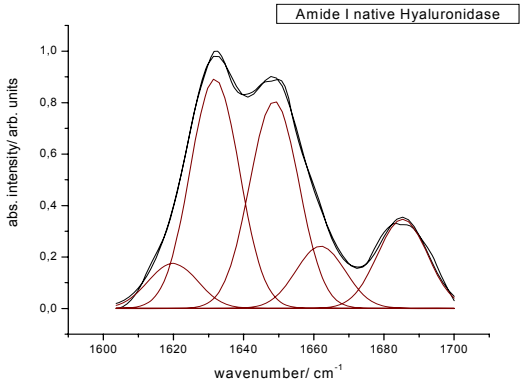
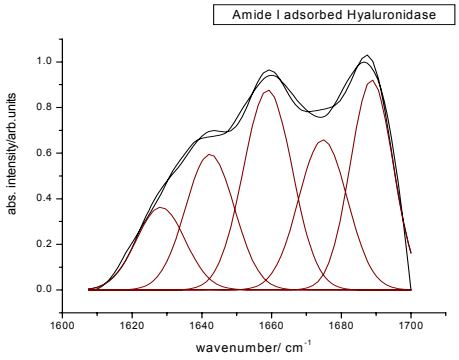


Fig. 3. a) ATR-FT-IR spectra of PMMA surface, b) Hyaluronidase adsorbed to PMMA surface after 24 h incubation.



a)



b)

Fig. 4. Deconvolution spectra of amide I band of native hyaluronidase, lyophilized (a), and adsorbed to PMMA surface after 24 h incubation (b).

4. Conclusions

FT-IR, FT-Raman and surface enhanced Raman spectra of hyaluronidase adsorbed on Ag colloidal surface have been obtained and discussed in order to get insight into the vibrational properties of the free and adsorbed species. SERS spectra of hyaluronidase on Ag colloidal nanoparticles revealed well resolved Raman signal of the chemisorbed species and allowed the presumption of oxygen-adsorption from the extreme functional groups of the complex molecular structure. The three N atoms contained in the secondary amides of the structure were sterically unable to bind to the Ag aggregates. Adsorption behaviour of the title species was further investigated within the PMMA bone substitute. ATR-FT-IR spectra of PMMA surface after incubation in protein solution indicate the adsorption of hyaluronidase to the biomaterial surface, emphasized by the presence of amide I and II bands.

Fourier deconvolution of amide I and band fitting procedure were performed in order to obtain more qualitative and quantitative information related to the adsorption induced conformational transitions, showing the decrease of α helix and β sheet content upon the adsorption to biomaterial.

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