

***In Vitro* Evaluation of the Sensitivity of a Hyaluronic Acid PEG Cross-Linked to Bovine Testes Hyaluronidase**

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Abstract

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Neauvia Intense is biocompatible, injectable hyaluronic acid (HA) filler PEG cross-linked for facial soft-tissue augmentation that provides volume to tissues. The aim of the present study is to evaluate the sensitivity of Neauvia Intense in hyaluronidase from bovine testes in a time-course analysis. The test is based on the colourimetric determination of the N-acetyl - D - glucosamine (NAG) released by the hyaluronidase in standardised conditions. The in vitro conditions involve the treatment of Neauvia Intense with a known concentration of the enzyme (6080U/ml). The NAG content was determined at different times to assess the kinetics of the degradation (1h, 3h, 6h, 24h, 48h, 72h, 120h, and 168h); the Ehrlich's reagent was used for the colourimetric quantification, by the method described by Reissing and colleagues. The intensity of the violet colour developed after the chemical reaction was proportional to the NAG present in each sample. A microplate reader at 585 nm read the absorbance. The amount of NAG released by the product was proportional to the time of incubation with bovine hyaluronidase, reaching a plateau after 168 hours.

Introduction

Hyaluronic acid (HA) is a high-molecular-mass linear anionic polysaccharide without branching side - chains, composed of 2000 - 25000 disaccharide units formed by glucuronic acid and N - acetyl - D - glucosamine (NAG), which are linked by $\beta(1,4)$ -glycosidic bond, reaching 105 - 107 Da in molecular mass [1][2][3][4][5]. HA is a major component of the extracellular matrix in the human body, as it is present

in the skin, synovial fluid, loose connective tissues, umbilical cord, vitreous body of the eye and cartilage [6] Several biological functions are fulfilled by this molecule, as it plays a major role in the organization and integrity of the extracellular matrix, thereby participating in the preservation of the form and the spatial arrangement of tissue components. It is also involved in numerous biological and physiological functions, such as cell motility, cell-matrix adhesion, and cell proliferation, water homeostasis of tissues or joint lubrication [7]. Thanks to the HA molecule's capability to absorb a large volume of water, it can

hydrate tissues and finally to maintain the moisture of the skin [8][9].

The degradation of the HA molecule occurs in the body by three mechanisms: the attack by free radicals, enzymatic processes (hyaluronidases) or thermally [10]. The hyaluronidases are endoglycosidases that cleave HA, reducing its viscosity [11]. Hyaluronidases could be classified into three groups according to their mechanism of action and end products: mammalian hyaluronidase (testis tube), leech/hookworm hyaluronidase and microbial hyaluronidase [12][13]. Mammalian and microbial hyaluronidases act on the β -1, 4 - glycosidic linkages of HA, while the leech/hookworm hyaluronidase degrades the β -1, 3 - glycosidic bond; all of them degrade the HA molecule and produce oligosaccharides of different chain length [14][15]. These enzymes are normally employed for several purposes, such as the prevention of tissue damage after the extravasation of different substances, for oedema reduction [16] and to prevent the diffusion of several substances injected subcutaneously [17][18][19][21].

In the last decade, HA has been considered, in aesthetic medicine, as an ideal substance to be used to augment the skin volume, being highly biocompatible, non-immunogenic and capable of binding water to a large extent [22]. In order to increase the biomechanical properties and the resistance to enzymatic break down of natural HA containing preparations, various methods have been developed for chemical modification or cross-linking of native HA into gels by covalent links, resulting in larger and most stable derivatives that retain HA biocompatibility and biodegradability in vivo [23][24].

In the HA hydrogel evaluated the crosslinking reaction occurring with cross-linking agents based on epoxides, in a strongly basic environment with the formation of ether bonds C - O - C, which is among the most solid and consequently the most resistant to degradation.

The reaction consists in the epoxide ring opening, in the simultaneous deprotonation of the hydroxyl group (in a basic environment is more nucleophilic than the carboxylic deprotonated group) and in the formation of the bond C - O - C.

The crosslinker used for this type of reaction to create Neauvia Hydrogel was PEGDE (Figure 1).

In aesthetic medicine, hyaluronidases are used for dissolving the injected hyaluronic acid filler, eliminating nodules, correcting the injection of excessive quantities of injected filler, or avoiding ischemic complications derived from this practice [25][26]. The literature reported a wide range of techniques to analyse the in vitro degradation rate of crosslinked HA hydrogels by the hyaluronidase enzyme [27][28]: change in viscosity, in water content, gel plate assay in Petri dishes or colourimetric assays

for the released glucuronic acid.

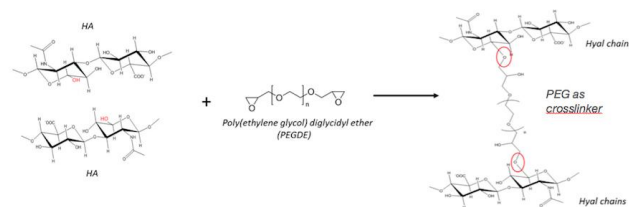


Figure 1: Hyaluronic acid (HA) crosslinking with poly(ethylene glycol) diglycidyl ether (PEGDE)

The aim of the present work is to evaluate the sensitivity to a bovine hyaluronidase enzyme of Neauvia Intense® (MatexLab SA, Lugano, CH), a product composed of pure hyaluronic acid of probiotic origin (*Bacillus Subtilis*) PEG cross-linked (polyethylene glycol) [29][30][31].

Materials and Methods

Chemicals and instruments

The sensitivity of Neauvia Intense® (MatexLab SA, Lugano, CH) to hyaluronidase was tested. This product is composed of pure hyaluronic acid of probiotic origin (*Bacillus Subtilis*) PEG cross-linked (polyethylene glycol) to obtain a 3D hydrogel matrix, that could be considered a completely biocompatible and degradable hydrogel, with volumizing effect, typical of the HA filler cross-linked polymer

Type I - S hyaluronidase from bovine testis was purchased from Sigma Aldrich (ref. H3506, 451Units/mg); all other chemicals were of the highest purity available.

The absorbance was measured using a Multiskan - Go (Fisher Scientific) spectrophotometer.

Sample preparation

Neauvia Intense® was weighed (0.2g) and placed in the bottom of glass tubes. The tubes were then centrifuged for 5min at 1000g in a refrigerated bench centrifuge (Megastar 600R, VWR) equipped with a swinging bucket rotor. At the end of the centrifugation, thin pellets firmly attached at the bottom of the tubes were obtained.

Hyaluronidase sensitivity test

To measure the degradation rate of the hyaluronidase on hydrogels, a Type I - S hyaluronidase from bovine testes (Sigma Aldrich; ref.

H3506, 451 Units/mg) was prepared at the concentration of 6080U/ml in an isotonic phosphate-NaCl buffer at a pH of 7.4 [2]. The glass tubes containing the gel pellets and the hyaluronidase solution were pre-incubated separately for 10min at 37°C. Then, 100 µl of the enzyme solution was added gently onto the surface of the gels and, after the incubation for 1h, 3h, 6h, 24h, 48h, 72h, 120h and 168h the enzymatic reaction was stopped by the addition of 0.1ml of a potassium tetraborate solution (0.8 mol/L, pH 9.1), followed by a stirring with a vortex mixer and heating for 3min at 100°C. The tubes were then cooled at room temperature, and the released NAG was assayed.

Assay of the released NAG

The measurement of the released NAG was performed according to the Reissig et al. method [1]. Briefly, a diluted 1:10 Ehrlich's reagent (Sigma Aldrich) in acetic acid was added to the tubes. The samples were vortexed and incubated for 20min at 37°C, to develop a violet colour, proportional to the released NAG content in each sample. The tubes were centrifuged at 1000 g for 15min to remove the gel fragments and the turbidity in the reaction. Then, the samples were read with a microplate reader (Multiskan-Go, Fisher Scientific) at 585 nm wavelength against the blank prepared with the only phosphate buffer and the Ehrlich's reagent.

Results

Hyaluronidase sensitivity test

To measure the degradation rate of Neuvia Intense, the product was weighed and incubated with a constant concentration of hyaluronidase enzyme. The amount of NaHA (mg) contained in the weighed product was reported in Table 1.

Table 1: Amount of NaHA (mg) contained in each sample. The indicated values correspond to the mean of at least two measurements ± SD for each time of analysis

NaHAcontent (mg/g)	Time	Weight (g) sample	NaHA (mg) amount in the sample
28	1h	0.208±0.002	5.810±0.048
28	3h	0.204±0.010	5.707±0.0273
28	6h	0.204±0.007	5.705±0.188
28	24h	0.197±0.004	5.516±0.112
28	48h	0.201±0.003	5.614±0.090
28	72h	0.205±0.003	5.734±0.084
28	120h	0.207±0.001	5.793±0.020
28	168h	0.202±0.000	5.662±0.000

The results of the time - course degradation of crosslinked HA by hyaluronidase are reported in Table 2.

Table 2: Absorbance values obtained after incubation of the samples with hyaluronidase and the relative amount of NAG (mg) released. The indicated values correspond to the mean of at least two measurements ± SD for each time of analysis

Time	Abs (585nm)	Abs per g of NaHA	total mg of NAG released	mg NAG/g released
1h	0.117±0.020	0.020±0.003	0.350±0.059	1.687±0.281
3h	0.181±0.050	0.032±0.008	0.540±0.141	2.657±0.713
6h	0.227±0.032	0.040±0.010	0.678±0.094	3.322±0.354
24h	0.312±0.083	0.057±0.004	0.933±0.247	4.735±1.245
48h	0.537±0.0105	0.096±0.020	1.605±0.315	8.001±1.552
72h	0.795±0.080	0.139±0.013	2.379±0.234	11.613±1.114
120h	0.867±0.074	0.150±0.012	0.593±0.221	12.529±1.027
168h	0.829±0.001	0.146±0.000	2.481±0.004	12.271±0.019

Results obtained after different times of incubation of Neuvia Intense® with a constant amount of hyaluronidase enzyme (Figure 2) showed an increase in the absorbance values (585 nm) concerning the blank sample.

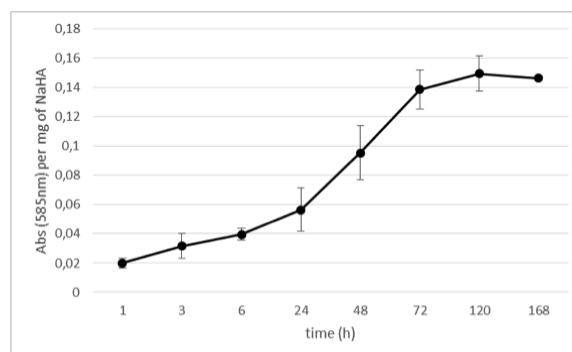


Figure 2: Absorbance values obtained after the incubation of Neuvia Intense® with the hyaluronidase enzyme for different times of treatment. Gel wt= 0.2g; Vol hyaluronidase = 100µl; Conc. hyaluronidase sample= 6080 U/ml;Temp= 37°C. Each point is the mean of at least two measurements ± SD

Interpolating these data with a standard NAG curve (data not shown), the amount of NAG (µg) released per g of product was calculated (Figure 3).

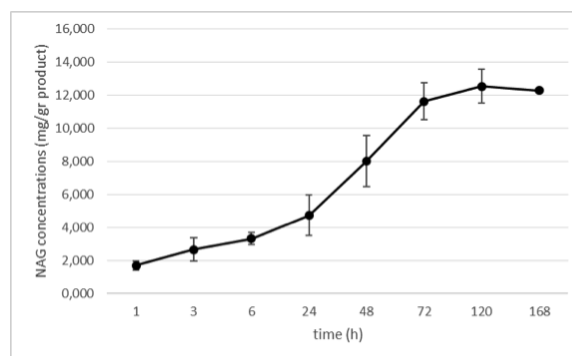


Figure 3: NAG amount (mg/g) released after different times of incubation of Neuvia Intense® with the hyaluronidase enzyme. Gel wt= 0.2g; Vol hyaluronidase = 100µl; Conc. hyaluronidase sample= 6080 U/ml;Temp= 37°C. Each point is the mean of at least two measurements ± SD

It is possible to note that the amount of NAG released increased with time and eventually levelled off from 120h of incubation on, suggesting complete digestion of the initial NaHA content in the sample.

In this study, the sensitivity of the product Neuvia Intense to a hyaluronidase from bovine testes was assayed. The growing interest of the aesthetic medicine sector in the use of such enzymes to dissolve or correct the injected hyaluronic acid filler, also avoiding misplacement HA complications derived from this injection practice, has been the starting point to deepen the mechanisms of action of the hyaluronidase on the Neuvia Intense product.

In detail, the method has been set up defining a determined quantity of product that was incubated with a constant concentration of hyaluronidase (6080 U/ml) at 37°C for a period ranging from 1 h to 168 h. The amount of NAG released, evaluated by the use of the Ehrlich's reagent, was calculated from the absorbance values measured at determined times of analysis and was considered as the factor corresponding to the degradation rate of the hydrogel caused by the enzymatic digestion process. The calculation was based on the interpolation of the absorbance values obtained from each sample with the values obtained for the standard NAG curve in µg. To this end, the centrifugation step of the final medium reaction before the absorbance measurement was necessary to remove the turbidity and small gel fragments still present in the tube [32].

The obtained results showed that the product Neuvia Intense, put in contact with a constant concentration of hyaluronidase enzyme, released a NAG quantity directly proportional to the incubation time and reached a plateau (maximum observed absorbance value) after 120h, monitored until 168h of contact with the enzyme. This has been considered the evidence that the hyaluronidase enzyme reached the highest levels of NAG release and consequently the maximum level of degradation of the HA molecule contained in the hydrogel.

The obtained results demonstrated that the Neuvia Intense product is sensitive to the bovine testes hyaluronidase, by a time course analysis that examined the rate of degradation of the HA molecule present in the hydrogel. Moreover, it is possible to conclude that the hyaluronidase brought to a complete degradation of the amount of Neuvia Intense product, in the experimental conditions performed, after 120h of treatment with the enzyme in an in vitro assay.

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