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Nano Particle CORONA Formation; Rather than Proteins

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Corona is a result of biological molecules and nanoparticles tending to each other and mainly refers to the case of proteins. The formation of non-protein corona; *i.e.*, carbohydrate and amino acids, and identifying the variations in their biological behavior were the purposes of this paper. The current study focused on silver nanoparticles (AgNPs, 20 and 120 nm), interaction with model small biomolecules, monosaccharides (glucose and fructose), and amino acids (histidine, cysteine, and tryptophan). The study considered the formation and composition of the corona as well as the characteristics and variations of carbohydrates/amino acids affected by AgNPs. Coronas were synthesized using the chemical reduction method, and their interactions with small biomolecules were monitored using response surface methodology (RSM). The results showed that treating glucose with smaller nanoparticles (20 nm) caused an increase in their size, agglomeration and aggregation, and a decrease in their homogeneity range. In the case of larger nanoparticles (120 nm), the glucose treatment caused to increase the size to ≥1 µm. Unlike glucose, fructose treatment had no influence on the size or stability of AgNPs. In the case of amino acids, cysteine, (containing the soft element of sulfur), and tryptophan (with the symmetry of electron distribution and no desire for an electron) had the strongest and weakest influence on AgNPs, respectively. Biological studies suggest that the very considerable influence on the properties of glucose in the AgNPs environments is growing. Corona formation influences the chemical properties as well as the biological features of biomolecules. *Pichia Pasturis* GS115 (a cysteine-deficient microorganism using glucose-containing growth media) in a medium containing untreated glucose and histidine treated with AgNPs, as well as the medium containing glucose treated with AgNPs and untreated histidine showed the least growth. The results may help the AgNPs toxicology and underlying biological mechanisms.

Keywords: Amino acid, Corona, DLS, Experimental design, HPLC, Monosaccharide, Silver nanoparticle

INTRODUCTION

In the 21st century, a keen interest arose in the development of nano-materials because of their unique properties [1]. Among metal nanoparticles, silver nanoparticles (AgNPs) grabbed special attention in many major applications in the medical prevention and treatment field based on their antibacterial features [2,3]. Even though there are various benefits of AgNPs, there is also the problem of toxicity [4-14]. The toxicity of AgNPs depends on many factors, including size, shape, and surface charge [5]. It is proposed that when NPs enter a cell or biological fluid, they are surrounded by biomolecules, mainly proteins [5].

The surface of nanomaterials in a biological environment is surrounded and covered by biomolecules. This coverage or crow is called a corona [6,15-17]. Coronas result from biomolecules and NPs tend to each other [18]. One well-cited assertion in the literature on coronas is that they are constructed according to different affinities of proteins to NPs [6,19]. Many studies have focused on the characteristics and formation of protein coronas. Despite the importance of lipids and carbohydrates, there is no information on how NPs interact with them [6,19,20].

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Typically, there is a big attraction of some public and/or some very specific biomolecules to bind to the surface of the NPs. Coronas are complex and multi-layered, composed of two male parts. They are constructed with an outer (soft) and an inner (hard) layer which differ in the exchange rate and power connector (binding strength) of biomolecules to the surface of the NPs [20-22].

In addition to macromolecules (proteins), small biomolecules such as amino acids and monosaccharides play a key role in preserving existing creatures. Interference with the function of macromolecules generally causes interference with the activity and control of the living system. Interference with the structure and function of small biomolecules may lead to fundamental problems [23]. The study considered the formation and composition of the corona as well as the characteristics and variations of carbohydrates/amino acids affected by AgNPs.

Materials and Method

All Chemicals were purchased from Sigma Aldrich Co. (Darmstadt, Germany). In all processes HPLC grade water used. The previous reported methods, with some modifications, were used to prepare AgNPs colloid [24]. In brief, $AgNO_3$ (1.0×10^{-3} M) aqueous solution containing 8% polyvinyl alcohol (PVA; MW: 88.000 Da) as a surfactant should be reduced using a drop wise addition of 3-sodium citrate solution (8% w/v) at near the boiling point (97 °C ± 0.5). In 15 min, yellowish grey colloids would be obtained. The AgNPs colloids [24] was investigated before and after interaction by UV-Vis spectroscopy (2501 PC UV-Vis, 0.1 cm quartz cuvette, 300-700 nm detection range; Shimadzu Co., Kyoto, Japan) and dynamic light scattering (DLS Nanophox equipped with 632.8 nm He Ne-laser, Sympatec Co., Germany [25]. Briefly, colloidal AgNPs with a spherical morphology had a maximum absorbance (λ_{max}) of 430 nm. Their size were optimized for 20.65 ± 1 nm (X99) and 120.21 ± 3 nm (X99) with homogeneity within the range of 0.1-1000 nm.

To investigate the AgNPs interaction with monosaccharide/amino acids, the Design Expert 7.0.1 software, distance-based response surface method (RSM) was used. The interaction time (10, 13, 16, 19, 25, 28, 43, 47, 65, 83, 102, 105, 108, 111 and 120 min) as a numeric factors in fifteen levels and two categorical factors including size of the nanoparticles (20 and 120 nm) and type of the biomolecule (glucose, fructose, histidine, cysteine and tryptophan) were designed, (Table 1). A total of 35 tests including 25 original modelling tests, 5 tests to assess the lack of fit and 5 margins testing and modelling were in the reduced cubic design. Drawings done to enhance the accuracy and reduction of system error and wrapped combination were carried out.

A Knauer HPLC system with a binary pump (model K-1001), and a K-2800 diode array detector (DAD), Knauer Co., model (Berlin, Germany), were used for chromatographic analysis. The detection wavelength was set at 200 nm. A 20 µl sample was injected into the analytical HPLC column for analysis. Amino acids was eluted and separated using mobile phase A (tris-buffer at the pH of 7.5) and mobile phase B (methanol) on Eurospher C18 (5 lm, 4.6 × 250 mm) at 30 °C. It starts with mobile phase A which decreased into 98% in 8 min and turns into 100% in 10 min and finished in 12 min (Fig. 1B). Monosaccharides were separated on YMC-PACK Polyamine II (250 × 4.6 mm, S-54 m, 12 nm) with isocratic elution of water 25:75 acetonitrile [23]. The flow rate of the mobile phase in the HPLC runs was set at 0.5 ml min^{-1} (Fig. 1A). Interaction percentage based on HPLC response is the normalized percentage of each peak area into the highest peak area (Run NO. 24).

In addition to physiochemical properties, the interaction influences on the biological features of glucose and histidine were investigated using histidine deficient GS115 *Pichia Pasturis* (purchased from Iranian Research Organization for Science and Technology (IROST)) growth on yeast nitrogen base medium (YNB). A GS115 *Pichia Pasturis* has a mutation in the histidine dehydrogenase gene (his 4) which prevents the synthesis of histidine. For this reason, it does not grow on the histidine deficient culture medium. Glucose is also one of the constituents of the YNB culture medium, and its influences over the AgNPs interaction could be studied.

RESULTS AND DISCUSSION

Silver nanoparticles (AgNPs) in biological environments interact with proteins [6], and their interactions result in the creation of a protein layer around AgNPs called a corona [6,

Table 1. Distance-based Design Methodology Design for the Study of Corona Formation and the HPLC Results

Std	Run	A: Time (min)	B: Size (nm)	C: Biomolecule	Interaction percentage based on HPLC response
23	1	111	120	Fructose	5.39590
30	2	65	20	Glucose	22.45678
21	3	16	120	Tryptophan	1.06730
14	4	120	20	Tryptophan	0.76565
7	5	65	20	Tryptophan	0.06293
29	6	10	120	Buffer	0.07317
9	7	65	120	Buffer	0.16078
34	8	120	120	Glucose	23.92718
1	9	10	20	Glucose	32.49550
22	10	120	120	Buffer	0.08745
15	11	102	120	Tryptophan	0.67391
4	12	25	120	Fructose	4.92114
35	13	83	20	Fructose	4.28381
28	14	65	20	Buffer	0.04045
16	15	19	20	Buffer	0.09541
6	16	47	20	Histidine	87.44469
24	17	13	120	Cysteine	86.85402
11	18	47	120	Glucose	22.54205
27	19	65	120	Tryptophan	0.67239
2	20	120	120	Glucose	22.27660
12	21	28	20	Histidine	92.96445
19	22	10	120	Histidine	90.45351
17	23	13	20	Fructose	5.14062
25	24	108	20	Cysteine	100.00000
10	25	111	20	Glucose	22.33807
33	26	47	20	Histidine	88.57260
32	27	83	120	Cysteine	92.74969
20	28	10	20	Tryptophan	0.36766
3	29	83	20	Fructose	5.18738
31	30	25	120	Fructose	4.95722
8	31	102	20	Buffer	0.09094
18	32	83	120	Cysteine	94.55159
13	33	105	20	Histidine	98.11655
5	34	120	120	Cysteine	86.89955
26	35	43	20	Tryptophan	0.52078

Table 2. ANOVA for the Study of Corona Formation and the HPLC Results

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	
Model	332.1545	18	17.48182	564.3481	< 0.0001**	Significant
A-time	6.03E-05	1	6.03E-05	0.001945	0.9654	
B-size	0.057993	1	0.057993	1.872136	0.1914*	
C-Biomolecule	329.8226	5	65.96451	2129.467	< 0.0001**	
AB	0.034599	1	0.034599	1.116936	0.3073	
AC	0.341974	5	0.068395	2.20792	0.1077*	
BC	0.129841	5	0.025968	0.838307	0.5429	
Residual	0.464655	15	0.030977			
Lack of fit	4.351E-03	10	4.351E-04	0.005258	0.9198	Not significant
Pure error	0.029501	5	0.0059			
Std. Dev.: 0.976003		R-Squared: 0.978603		Adj R-Squared : 0.956834		

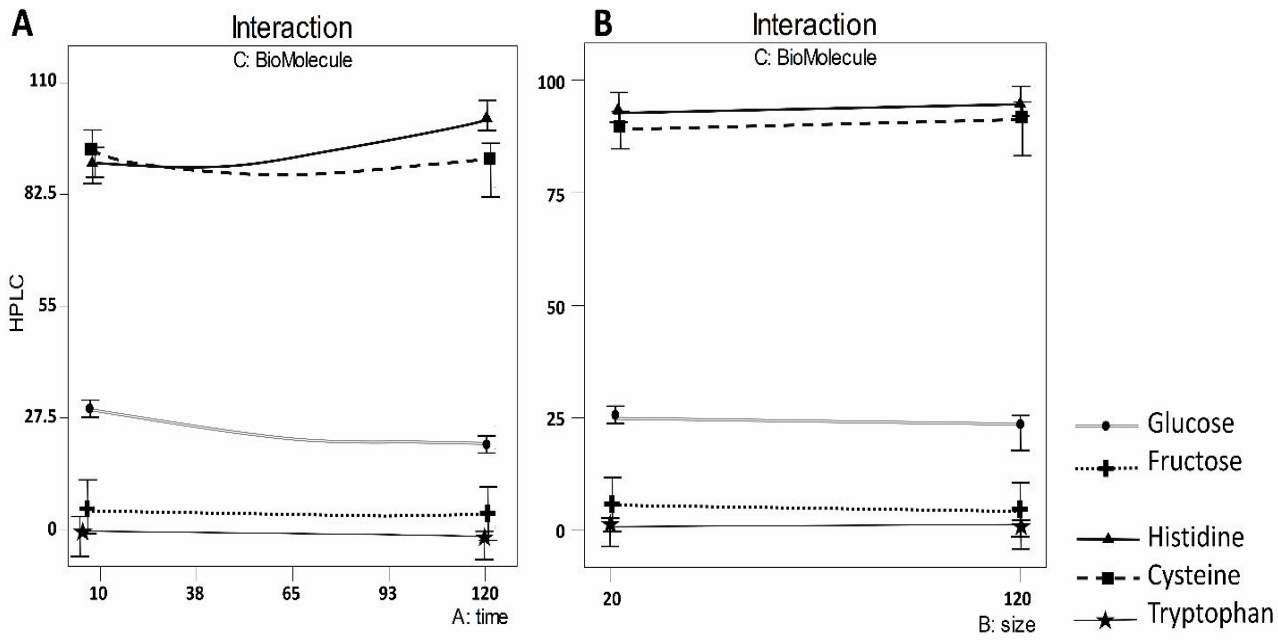

Fig. 1. Bio-molecules variation pattern by the generated model considering the variations of the effective parameters: (A) time (10-120 min), and (B) the size of AgNPs (20 and 120 nm).

20,21]. The physical, chemical, and biological characteristics of both AgNPs and proteins change this interaction. Although there are many reports of protein coronas, there are none on the small biomolecules and carbohydrate corona formation and features. Among carbohydrates, glucose and fructose were chosen, as they are the most important and vital monosaccharides [23]. In 2004, Li *et al*., who had studied the interactions between amino acids and AgNPs, reported that cysteine, tryptophan, and histidine have the most impressive interactions [26]. They were chosen for corona formation and biological variations in the current study. The increase in size and size distribution of AgNPs causes many variations in corona formation and stability [4,6,13,14,22]. AgNPs smaller than 30 nm and larger than 100 nm have different and unique features [18-20]. In this study, the interactions between AgNPs (20 and 120 nm) and monosaccharides (glucose and fructose) and amino acids (histidine, cysteine, and tryptophan) were evaluated through 35 designed experiments, and the results are shown in Table 1. In each experiment, the AgNP and their biomolecule coronas were separated using centrifuge (30 min × 22000 g) after the interaction time, and the amount of non-interacted biomolecules (monosaccharides or amino acids) were studied using the HPLC method. As can be seen in Table 1, cysteine and glucose had the greatest interaction ratio with 20 nm AgNPs in ~100 and ~30 min, respectively.

The ANOVA study of the results are shown in Table 2. The F-value in this table is the ratio of mean-squared error (MS) or each treatment to the residuals, obtained by repeating the experiment at the design centre point. The implication of the F-value depends on the degree of freedom of the model.

The effects with the F-probability lower than 0.0001 are considered to be statistically significant. The values of R^2 and adjusted R^2 were 97.86 and 95.68%, respectively. The standard error of the estimate showed a 0.9760 value for the standard deviation of the residuals. Inspection of the results showed that the main effects of bio-molecule type and AgNPs size, and the interactions between the time and bio-molecule type, were the most important parameters. Figure 1 shows the response interaction pattern by the generated model considering the variations of the effective parameters.

It can be seen from Fig. 1A that as the time increases the availability of Histidine and Cysteine increases and Glucose decreases. However, no spatial changes occur into Fructose and Tryptophan. Figure 1B shows that longer interaction time would cause more variations on bio-molecules/AgNPs features. Also, at long durations with each bio-molecule, the amounts of their availability were reduced.

The interaction caused changes in the colloidal solution color (Fig. 2). Glucose showed the greatest variations and may have more impact on the size/homogeneity and spectroscopic characteristics of AgNPs. The treatments performed using 20 nm AgNPs showed noticeable variations on the remaining monosaccharides. As can be seen in Fig. 2D, the interaction between glucose and AgNPs (120 nm) resulted in size increments and the broadening of size distribution. In the case of 20 nm AgNPs, the glucose treatment not only increased the size, but also drastically changed the homogeneity.

In these two types of glucose/AgNPs interactions, UV-Vis outcome (Fig. 2C) demonstrates that the AgNPs (120 nm)/glucose treatment is resulted in a red shift or bathochromic effect, while the AgNPs (20 nm)/glucose treatment is resulted in a hypochromic shift. In a nutshell, the DLS and UV-Vis (Figs. 1C and D) showed that the glucose treatment formed a corona, caused a size increment in AgNPs (120 nm), and altered the size distribution in 20 nm. It can be concluded that aggregation/agglomeration is more likely in the AgNPs (20 nm)/glucose treatment. It should also be noted that the samples were significantly different in terms of sustainability. The samples treated with fructose had a stable colloidal system until the end of the experiment in both 120 and 20 nm treatments, while the glucose treatments caused faster precipitation.

Among the chosen amino acids, cysteine containing sulphur (a soft element) had a high tendency to interact with Ag [27,28]. Also, the interaction of a positively charged amino acid, histidine, and negatively charged AgNPs (PVA surface modification) was studied. These two analyses can clarify whether or not the surface chemistry of AgNPs or its inherent nature is important. The results (Tables 1 and 2) demonstrated that histidine is more likely to interact with AgNPs than cysteine. Thus, it can be hypothesized that the surface chemistry of interacting constituents is much more important than their inherent nature in interaction of AgNPs

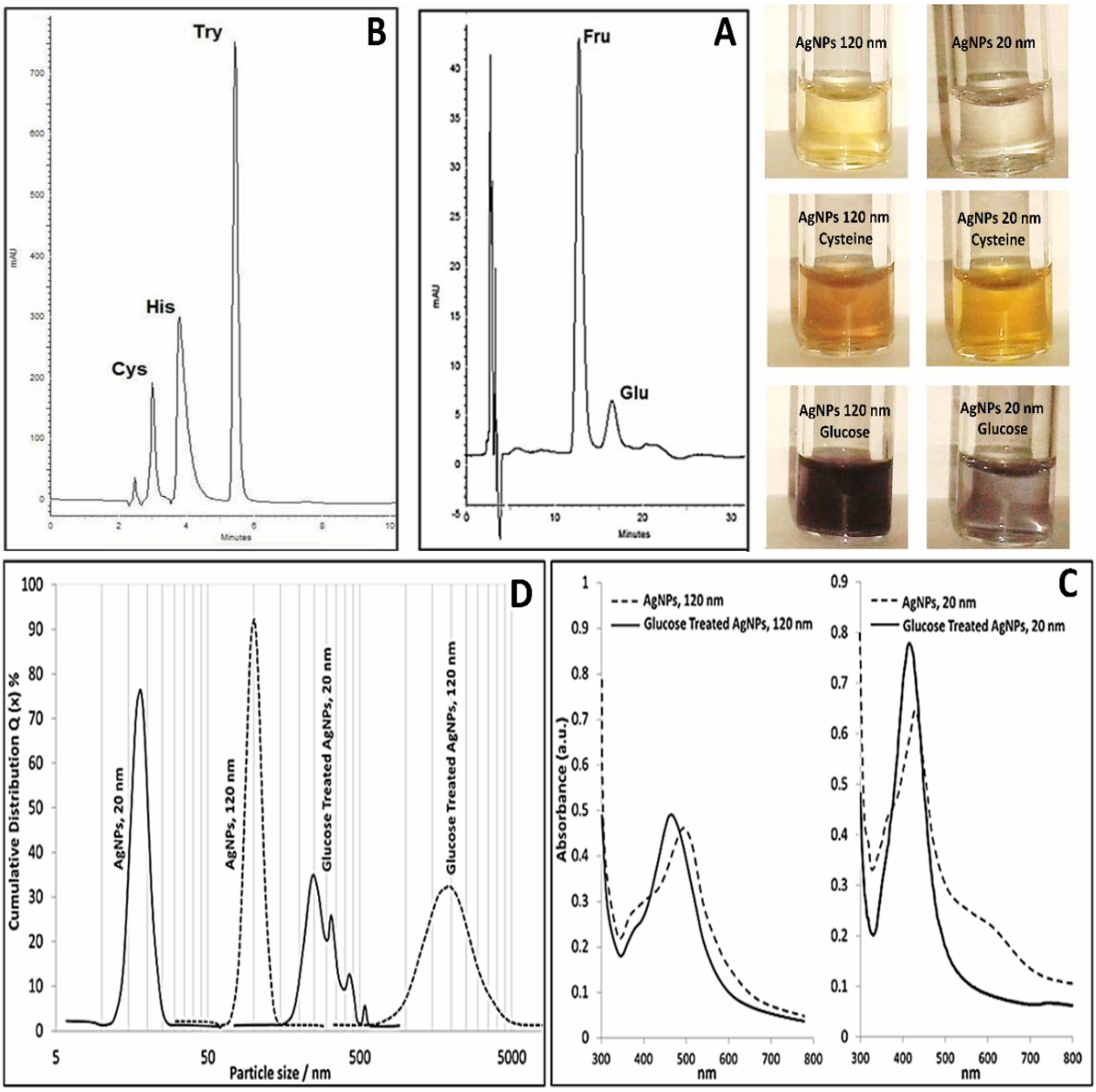

Fig. 2. HPLC chromatogram of (A) glucose (Glu) and fructose (Fru), and (B) histidine (His), cysteine (Cys) and tryptophan (Try), the UV-Vis (C) and DLS (D) analyses of AgNPs in the size of 20 and 120 nm before and after treatment with glucose.

with biomolecules. This was more specifically proven by the study of tryptophan, an aromatic amino acid with electron superconductivity, which had less interaction with AgNPs.

In addition to the physiochemical study of the interaction between AgNPs and glucose and histidine, its (20 nm) influence on the bioactivity of glucose and histidine was investigated on GS115 *Pichia Pasturis. P. Pasturis*

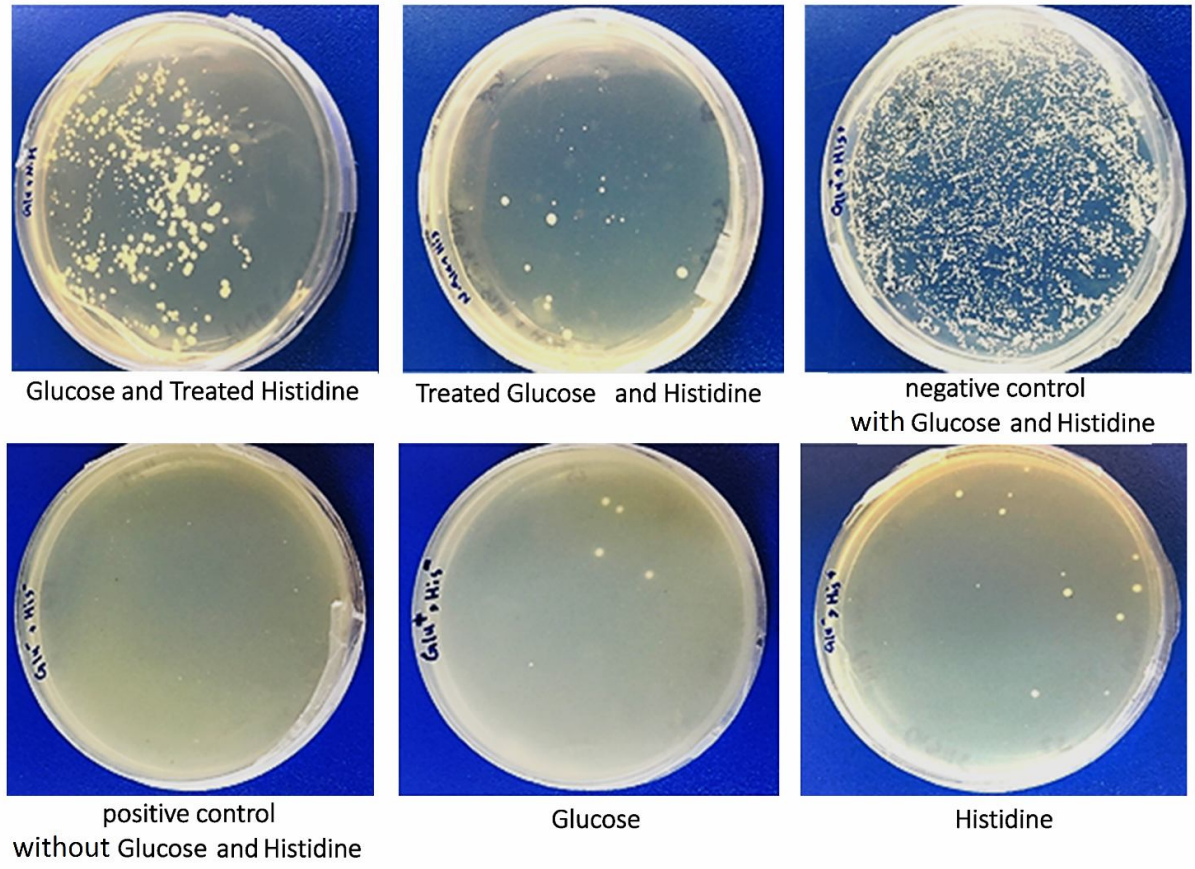

Fig. 3. Growth of GS115 *Pichia Pasturis* in the sterilized YNB-agar plate in different conditions.

GS115 is a candidate for this study because of its ability to grow in simple medium (Yeast Nitrogen Base [YNB], Sigma Co., Germany), being a convenient mammalian expression model system [29,30] and a mutated species which is unable to produce cysteine. YNB is suitable for the cultivation of yeast and its carbon source is glucose, so, it would be a good setting for this research. Figure 3 shows the ability and growth profile of *P. Pasturis* GS115 under the different treatments of glucose and histidine with AgNPs (20 nm). The negative control was its growth in the YNB agar media containing untreated glucose and histidine, and the positive control was the same growth media without glucose or histidine. As seen in Fig. 3, the YNB agar medium containing histidine and AgNP-treated glucose have very low growth compared to the one containing glucose and AgNP-treated histidine. The results indicate that the glucose treatment is more effective than the histidine treatment. Growth in the presence of glucose or histidine alone is also very limited, and both are necessary for growth.

CONCLUSIONS

This study shows the contribution of nanoparticles size into the characterization of coronas. When smaller nanoparticles were placed adjacent to glucose, they increased the corona diameter and its accumulation. In contrast, nanoparticles with a larger diameter exhibited a smaller diameter increase. The nature of carbon sources was also very effective in interacting with nanoparticles. Fructose, similar species to glucose, showed no response to the nanoparticles in terms of resizing and stability. Another

important challenge observed in this study was the formation of coronas which was completely dependent on the chemical properties of the surface of the nanoparticles and the biological molecule, and its biology characterization had a negligible effect on the formation of the corona. As explained, the amino acid cysteine, due to the presence of sulfur, has a high potential for binding and association with the silver group, the formation of a corona from this amino acid with nanoparticles of silver was much better than that from tryptophan and methionine. In the case of carbohydrates, the binding of glucose to the nanoscale silver was much better than the binding of fructose because of its specific spatial structure. All of these factors indicate that in forming an appropriate corona, the chemical characterization of superficial functional groups plays a much larger role in the nature of the biological material.

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