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ORIGINAL ARTICLE

Optimization of antimicrobial efficiency of silver nanoparticles against three oral microorganisms in irreversible hydrocolloid impressions

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ABSTRACT

Background and Objectives: Silver nanoparticles (Ag-NPs) are potent antimicrobial agents, which have recently been used in dentistry. The aim of the current study was to optimize antimicrobial activity of Ag-NPs used in preparing irreversible hydrocolloid impressions against three microorganisms of *Escherichia coli*, *Streptococcus mutans* and *Candida albicans.*

Materials and Methods: After assessing antimicrobial activity of the compound using disk diffusion method, three parameters of concentration of Ag-NPs (250-1000 ppm), ratio of hydrocolloid impression material powder to water (0.30-0.50) and time of mixing (20.0-60.0 s), affecting antimicrobial activity of irreversible hydrocolloid impression materials against the three microorganisms, were optimized. This combined process was successfully modeled and optimized using Box-Behnken design with response surface methodology (RSM). Decreases in colony number of *E. coli*, *S. mutans* and *C. albicans* were proposed as responses.

Results: Qualitative antimicrobial assessments respectively showed average zone of inhibition (ZOI) of 3.7 mm for *E. coli*, 3.5 mm for *S. mutans* and 4 mm for *C. albicans*. For all responses, when the mixing duration and powder-to-water ratio increased, the circumstances (mixing duration of 59.38 s, powder-to-water ratio of 0.4 and Ag-NP concentration of 992 response) increased. Results showed that in optimum ppm, the proportion of decreases in colony numbers was maximum (89.03% for *E. coli*, 87.08% for *S. mutans* and 74.54% for *C. albicans*). Regression analysis illustrated a good fit of the experimental data to the predicted model as high correlation coefficients validated that the predicted model was well fitted with data. Values of R2Adj with R2Pred were associated to the accuracy of this model in all responses.

Conclusion: Disinfection efficiency dramatically increased with increasing of Ag-NP concentration, powder-to-water ratio and mixing time.

Keywords: Dental impression materials; Nanotechnology; Microorganism

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INTRODUCTION

Contamination of dental impressions with the patient's blood and saliva, which are capable of transmitting infectious diseases to dental personnel, is a serious problem (1). Dental impressions carry a large number of potentially infective microorganisms found in saliva and blood (2). In dentistry, irreversible hydrocolloids or alginates are commonly used to record preliminary impressions (3). Because the set materials consist of a gel structure, they absorb water, particularly during the disinfection progress (3). Irreversible hydrocolloids (alginates) are the most commonly used impression materials and have been shown to be more vulnerable to infections than other impression materials such as silicone (4). Guidelines are issued by the American Dental Association (ADA) and American National Standards Institute (ANSI), which recommend spraying or immersing disinfectants based on the characteristics of the dental impressions. Compatibility, time of immersion and concentration and composition of disinfectants are the major factors, which can affect dental impressions (5). One of these disinfectants, silver, has been used for the treatment of wounds for centuries. Silver and its derivatives are used as antimicrobial agents, especially nanosilver products have been used in various galenic applications (5).

Nowadays, silver nanoparticles (Ag-NPs) have a broad use in medical products and equipment (6). Because of their effective antimicrobial characteristics and low toxicity to mammalian cells, nanosilver products have become one of the most popular nanomaterials in consumer products (7). Despite widespread uses of nanosilver products, a few studies have optimized effects of major variables on antimicrobial characteristics of irreversible hydrocolloid impressions incorporated with Ag-NPs (8-10). Therefore, the purpose of this study was to assess and optimize antimicrobial efficacy of Ag-NPs, used as a colloid solution in preparing irreversible hydrocolloid impressions against three common oral pathogens of *Escherichia coli, S. mutans* and *C. albicans.* To the best of the authors' knowledge, there were no published studies on optimization of antimicrobial activity of irreversible hydrocolloid impression materials incorporated with Ag-NPs. The hypothesis was that optimizing antimicrobial process of irreversible hydrocolloid impressions incorporated with Ag-NPs could improve its antimicrobial activity.

MATERIALS AND METHODS

Microorganisms and culture media. The microorganisms were provided by American Type Culture Collection (ATCC), including *Escherichia coli* ATCC 25922, *Streptococcus mutans* ATCC 35668 and *Candida albicans* ATCC 10231. Blood agar was used for *S. mutans*, tryptic soy agar (TSA) for *E. coli* and Muller-Hilton agar for *C. albicans*; all purchased from Merck, Germany.

Irreversible hydrocolloid impression materials. The irreversible alginate impression materials included Kromopan Class A Type I dust-free (Lascod, Italy), complying with ISO 1563.

Silver based nanoparticles. The colloidal forms (AgNPs) were commercially prepared by Nano Nasb Pars, Tehran, Iran.

Process variable and Box-Behnken design. The statistical design of experiments (DOE) was used to optimize three operating variables of the antimicrobial activity process of alginate incorporated with nanosilver solution, including concentrations of Ag-NPs, ratio of irreversible hydrocolloid impression powder-to-water and time of mixing of the irreversible hydrocolloid impression with nanosilver particles (Table 1). Process was optimization based on a statistical method called response surface methodology (RSM) to show performance of composite systems. Using Design Expert 11 Statistical Software, Box-Behnken design (BBD) with RSM was used to statistically optimize the antimicrobial process (Table 1). A total of 15 experiments were carried out to assess effects of the three significant independent parameters on the antimicrobial process. Results were analyzed using analysis of variance (ANOVA) and statistical response plot.

Table 1. Experimental parameters and their levels in Box-Behnken design.

Symbol	Factor	Range
A	Mixing duration (second)	20.0-60.0
B	Powder-to-water ratio	0.30-0.50
C	Ag-NP concentration (ppm)	250-1000

Statistical analysis and model validation. All

data generated from Box-Behnken experiment were subjected to multiple regression analysis using least squares to build the regression models. Experimental design, data analysis, interaction plotting and optimization of factor conditions were carried out using Design Expert 11 Statistical Software.

Antimicrobial testing. Based on Box-Behnken design matrix, each experiment was prepared as follows: the irreversible hydrocolloid impression material powder incorporated with each concentration of Ag-NPs (0.25, 0.50 and 1.00% w/v) was mixed with water following powder-to-water ratio of 0.3, 04 and 0.5 and poured into sterile Petri dishes. Test tubes containing nutrient broth were inoculated with the microbial suspensions and incubated at 35-37°C for 24 h. Microorganisms were recovered from frozen stock cultures and cultured in 10 ml of nutrient broth at 35-37°C for 24 h. To achieve 10^8 CFU/mL of each highlighted microorganism as indicator, 1 mL of the overnight suspension was mixed with 9 mL of the specific broth media. Of this suspension, a serial dilution was prepared to a final concentration of 10^5 CFU/ mL. Suspensions were refrigerated until use, no longer than 6 h.

Quantitative method. Quantitative antimicrobial activity of the Ag-NPs was assessed against the three microorganisms (8). Standard solutions were used as mixing solutions for each experiment. Generally, 2 mL of the suspensions were used for each microorganism using two sterile tubes. Irreversible hydrocolloids were prepared for each experiment and set at room temperature. Samples from each experiment were separately weighed and blended. Two grams of the irreversible hydrocolloid powder were added to each tube containing the microbial suspension with agitation. For each experiment, 100 µL of the inoculated suspensions were poured into a sterile Petri dish. The Petri dish was then filled with appropriate agar media and incubated at 35-37°C for 24 h. Results were reported after counting the microbial number for each sample. Proportions of decreases were calculated using the following formula of $100\frac{(B-A)}{B}=R$. Where, R was the decrease rate (%), A was the number of microorganisms recovered from the treated test sample during a desired contact time and B was the number of microorganisms recovered from the treated test sample immediately after inoculation (at zero contact time). Three responses were as follows: Response 1, decreases in colony numbers of *E. coli* (%); Response 2, decreases in colony number of *S. mutans* (%) and Response 3, decreases in colony number of *C. albicans* (%).

RESULTS

Quantitative result. Various experiments were carried out using BBD technique to investigate effects of the process variables on the efficacy of Ag-NP antibacterial ability against the three pathogens (Table 2).

Table 2. Box-Behnken design matrix with responses.

	Factors			Responses		
No.	**A**	**B**	**C**	**R_1**	**R_2**	**R_3**
1	40	0.5	250	52	42	38
2	40	0.3	250	51	41	37
3	60	0.4	1000	89	86	74
4	40	0.5	1000	81	80	67
5	60	0.5	625	71	63	56
6	20	0.4	250	53	43	36
7	20	0.5	625	61	50	42
8	60	0.3	625	68	55	51
9	40	0.4	625	70	60	49
10	40	0.3	1000	78	72	64
11	40	0.4	625	69	59	50
12	20	0.3	625	61	49	43
13	40	0.4	625	71	61	48
14	60	0.4	250	52	42	36
15	20	0.4	1000	72	67	53

No., number of the experiment; A, mixing duration (20-40 s); B, powder-to-water ratio (0.3-0.4); C, Ag-NP concentration (250-1000 ppm); R1, Response 1; R2, Response 2; R3, Response 3.

Analysis of variance. Results of the ANOVA for the model for each response are presented in Tables 3, 4 and 5. Importance of each parameter was determined using F-value and P-value. The F-value was the standard deviation (Std. Dev) of the mean value and the expressions of the model having P-value less than 0.05 were meaningful. In this model, all the parameter including values less than 0.05 were significant, except for parameters AB and BC in Response 1 and parameter BC in Response 3. A good non-fit model was expected low (18). Disparity with P-value of 0.9630 for Response 1 and 0.9630 for Responses 2 and 3 indi-

Table 3. Analysis of variance of quadratic Response 1 level.

Source	Sum of squares	DF	Mean of squares	F-value	P-value
Model	1841.35	9	204.59	454.65	< 0.0001
A	136.12	1	136.12	302.50	< 0.0001
B	6.13	1	6.13	13.61	0.0142
C	1568.00	1	1568.00	3484.44	< 0.0001
AB	2.25	1	2.25	5.00	0.0756
AC	81.00	1	81.00	180.00	< 0.0001
BC	1.00	1	1.00	2.22	0.1962
A2	12.98	1	12.98	28.85	0.0030
B2	30.52	1	30.52	67.82	0.0004
C2	9.75	1	9.75	21.67	0.0056
Residual	2.25	5	0.45	0.083	0.9630
Lack of fit	0.25	3	0.083	454.65	0.9630
Pure error	2.00	2	1.00		
Cor total	1843.60	14			

DF: degrees of freedom.

Table 4. Analysis of variance of quadratic Response 2 level.

Source	Sum of squares	DF	Mean of squares	F-value	P-value
Model	2761.75	9	306.86	681.91	< 0.0001
A	171.12	1	171.12	380.28	< 0.0001
B	40.50	1	40.50	90.00	0.0002
C	2346.12	1	2346.12	5213.61	< 0.0001
AB	12.25	1	12.25	27.22	0.0034
AC	100.00	1	100.00	222.22	< 0.0001
BC	12.25	1	12.25	27.22	0.0034
A2	23.08	1	23.08	51.28	0.0008
B2	39.00	1	39.00	86.67	0.0002
C2	14.77	1	14.77	32.82	0.0023
Residual	2.25	5	0.4500		
Lack of fit	0.2500	3	0.0833	0.0833	0.9630
Pure error	2.00	2	1.0000		
Cor total	2764.00	14			

DF, degrees of freedom.

cated that the second-order equation suggested for this project was valid (18).

Regression analysis. Statistical parameters verifying adequacy of the quadratic model are shown in Table 6.

A quadratic model was chosen to predict the response based on Table 6. The anticipated model was well fitted with data in this model, as evidenced by excellent correlation coefficients (0.9988 for Response 1, 0.992 for Response 2 and 0.9988 for Response 3). The correlation between R^2_{Adj} and R^2_{Pred} was in line with the model accuracy and their closeness to 1 indicated that the experimental and anticipated outcomes were in good agreement. Response 1 included an adequate precision of 69.37 > 4, Response 2 included an adequate precision of 82.38 > 4 and Response 3 included an adequate precision of 70.29 > 4, indicating good signal-to-noise ratios. The model low Std. Dev (0.67) reflected the model good accuracy (18).

Table 5. Analysis of variance of quadratic Response 3 level.

Source	Sum of squares	DF	Mean of squares	F-value	P-value
Model	1925.35	9	213.93	475.40	< 0.0001
A	231.12	1	231.12	513.61	< 0.0001
B	8.00	1	8.00	17.78	0.0084
C	1540.13	1	1540.13	3422.50	< 0.0001
AB	9.00	1	9.00	20.00	0.0066
AC	110.25	1	110.25	245.00	< 0.0001
BC	1.0000	1	1.0000	2.22	0.1962
A2	6.98	1	6.98	15.51	0.0110
B2	0.5192	1	0.5192	1.15	0.3318
C2	16.67	1	16.67	37.05	0.0017
Residual	2.25	5	0.4500		
Lack of fit	0.2500	3	0.0833	0.0833	0.9630
Pure error	2.00	2	1.0000		
Cor total	1927.60	14			

DF, degrees of freedom.

Effects of various parameters on the antimicrobial efficiency. Effective interactions between the initial concentration of Ag-NPs, irreversible hydrocolloid impression powder-to-water ratio and mixing duration on antimicrobial efficiency are demonstrated in Fig. 1 for Response 1, Fig. 2 for Response 2 and Fig. 3 for Response 3. For each response, three-dimensional (3D) was used to represent interactions between the parameters. For all responses, when the mixing duration and powder-to-water ratio increased, the response increased. Contour plots of the parameters also verified the 3D plots, with the highest response at mixing duration of 60 s and powder-to-water ratio of 0.5 (Fig. 1a; Fig. 2a and Fig. 3a). Based on Fig. 1b; Fig. 2b and Fig. 3b, positive effects of Ag-NP concentration and mixing duration on responses are clearly seen. The Fig. 1c; Fig. 2c and Fig. 3c repre-

Table 6. Statistical parameters verifying adequacy of the quadratic model.

Model	R^2_{Sqared}	R^2_{Adj}	R^2_{Pred}	PRESS	Std. Dev
Quadratic (Response 1)	0.9988	0.9966	0.9954	8.50	0.67
Quadratic (Response 2)	0.9992	0.9977	0.9969	8.50	0.67
Quadratic (Response 3)	0.9988	0.9967	0.9956	8.50	0.67

PRESS, predicted residual sum of squares; std. dev, standard deviation.

sent 3D plots of the effects of Ag-NP concentration and powder-to-water ratio parameters on the response. The other factor, mixing duration, was constant at its midpoints.

Optimization of the independent variables and validation of the experiments. Optimization of antimicrobial parameters such as Ag-NP concentration, powder-to-water ratio and mixing time was carried out using RSM. The best parameters for Ag-NP concentration, powder-to-water ratio and mixing time were 992 ppm, 0.4 and 59.38 s, respectively. When these parameters were set at their optimization points, the proportion of decreases in colony numbers was maximum (89.03% for *E. coli*, 87.08% for *S. mutans* and 74.54% for *C. albicans*) with an overall desirability value of 1 (Fig. 4).

DISCUSSION

Nanoparticles are particulate dispersions or solid particles having sizes of 10-100 nm (11). Recently, Ag-NPs have been popular because they are non-toxic to humans at low concentrations and include antibacterial characteristics within a broad spectrum (12). Indeed, Ag^+ ions and Ag^- based compounds are known to be harmful to microorganisms, with severe biocidal effects on at least 12 bacteria species, including multiple-resistant bacteria such as methicillin-resistant bacteria (13, 14). The mechanism of Ag^+ ion inhibitory effects on microorganisms is unknown; however, AgNPs interact with a wide range of molecular processes within microorganisms, resulting in a variety of effects from inhibition of growth to loss of infectivity to cell death, depending on shape, size and concentration of AgNPs and microbial sensitivity to silver (15, 16).

This study investigated the major factors affecting antimicrobial activities of irreversible hydrocolloid impression materials incorporated with Ag-NPs. The RSM was used to assess and optimize antimicrobial activities of the irreversible hydrocolloid impression materials incorporated with Ag-NPs. Effects of the three independent variables (Ag-NP concentration of 250-1000 ppm, powder-to-water ratio of 0.3-0.5 and mixing duration of 20-60 s) on three responses were investigated and the optimal conditions were check using Box-Behnken experimental design of RSM to improve decreases in colony number of *E. coli* (%), *S. mutans* (%) and *C. albicans* (%). Three independent process variables, including mixing duration (A), powder-to-water ratio (B) and Ag-NP concentration (C), significantly affected the antimicrobial efficiency and the optimal range of each tested variable was determined. Three repeats of the central run, leading to 15 sets of experiments, enabled each experimental response to be optimized. When the mixing duration and powder-to-water ratio increased, the response increased. This was correlated with the results of Ginjupalli et al. (2016), who suggested that increased concentration of Ag-NPs significantly increased their antimicrobial activities (17). Furthermore, addition of Ag-NPs to irreversible hydrocolloid impression materials such as Zelgan and tropicalgin resulted in better antimicrobial activities with no or minimum adverse effects on their characteristics (18). Antimicrobial activities of Ag-NPs against growth of *E. coli* in Luria-Bertani (LB) agar have been reported by Kim et al. (2009) (14). For 10^5 and 10^4 CFU *E. coli*, growth inhibitory doses of Ag-NPs were 50-60 and 20 g/cm^3, respectively. Antifungal characteristics of Ag-NPs against *C. albicans* have been reported in studies by Kim et al. (2009) (14). As demonstrated in Panacek's study, Ag-NPs have strong antifungal efficacy against pathogenic *Candida* spp. at concentrations of roughly 1 mg/L of Ag (15). The antifungal activity of Ag-NPs is equivalent to that of ionic silver; however, at concentrations that impede the growth of the studied yeasts, ionic silver remains cytotoxic (15). The Ag-NPs prevent yeast growth at very low concen-

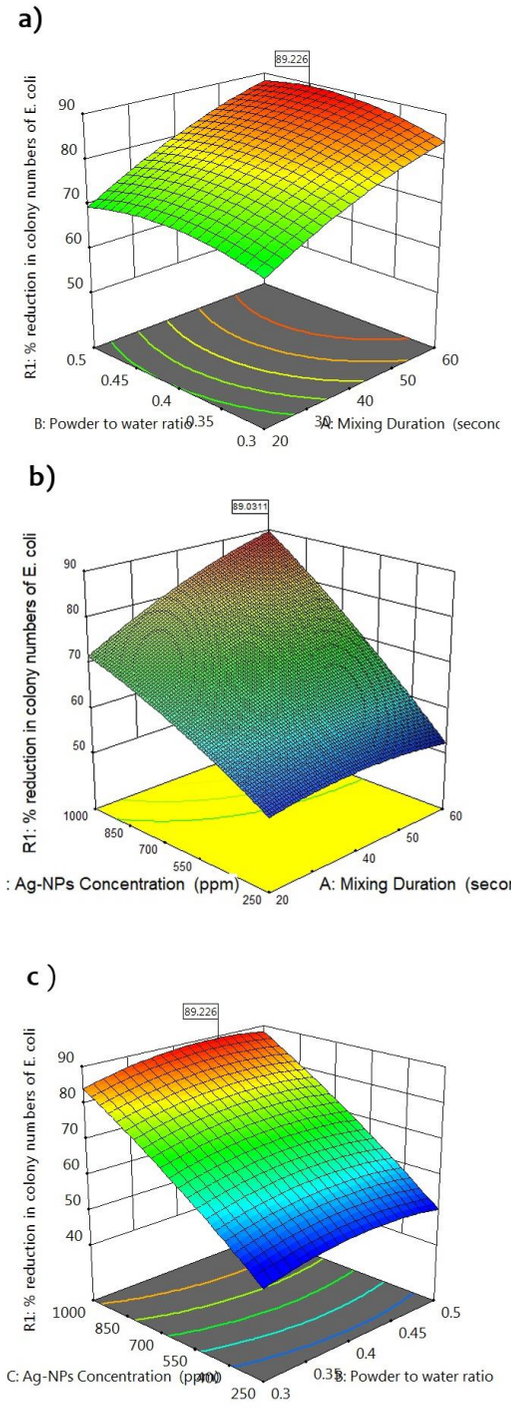

Fig. 1. 3D plot of effective interactions between the initial concentration of Ag-NPs, irreversible hydrocolloid impression powder-to-water ratio and mixing duration on decreasing proportions in colony numbers of *E. coli* (Response 1): a) effects of mixing duration and powder-to-water ratio parameters; b) effects of Ag-NP concentration and mixing duration parameters; and c) effects of Ag-NP concentration and powder-to-water ratio parameters.

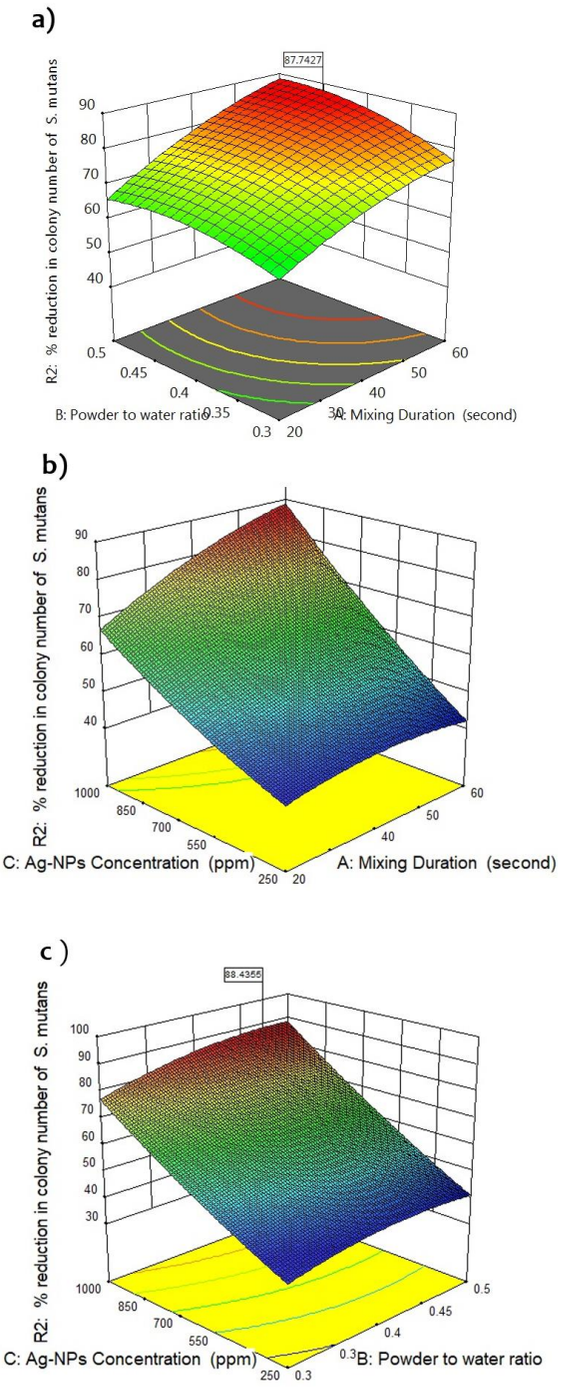

Fig. 2. Effective interactions between the initial concentration of Ag-NPs, irreversible hydrocolloid impression powder-to-water ratio and mixing duration on decreasing proportions in colony numbers of *S. mutans* (Response 2): a) 3D plot for the effects of mixing duration and powder-to-water ratio parameters; b) 3D plot for the effects of Ag-NP concentration and mixing duration parameters; and c) 3D plot for the effects of Ag-NP concentration and powder-to-water ratio parameters.

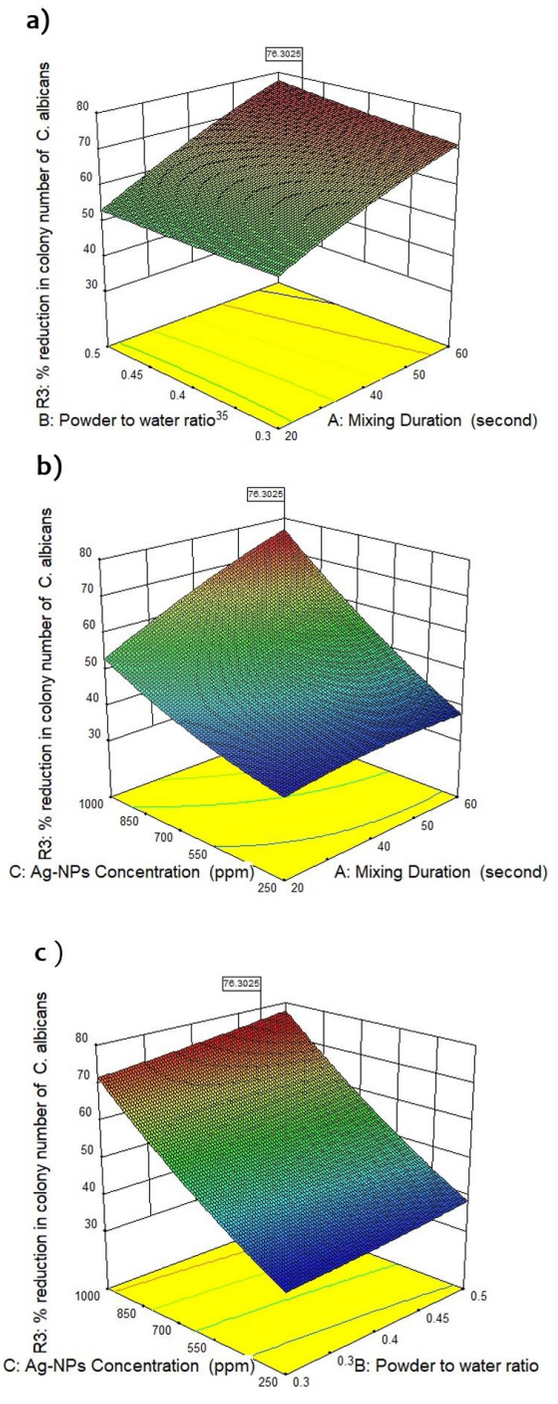

Fig. 3. Effective interactions between the initial concentration of Ag-NPs, irreversible hydrocolloid impression powder-to-water ratio and mixing duration on decreasing proportions in colony numbers of *C. albicans* (Response 3): a) 3D plot for the effects of mixing duration and powder-to-water ratio parameters; b) 3D plot for the effects of Ag-NP concentration and mixing duration parameters; and c) 3D plot for the effects of Ag-NP concentration and powder-to-water ratio parameters.

trations, comparable to those of typical antifungals (15). It may be concluded that silver NPs are effective antimicrobial agents against common pathogens based on their previously reported high antimicrobial activities (9). The MICs reported in prior studies of silver NP antifungal activity were not as low as those in this analysis (9). As demonstrated by Ginjupalli et al. (2016), adding Ag-NPs to irreversible hydrocolloids resulted in dose-dependent antibacterial activities (17). Antimicrobial characteristics of hydrocolloid impression materials incorporated with Ag-NPs against *S. aureus* have been reported by Ginjupalli et al. (2016) (17). Based on these studies, $AgZrPO_4^-$ containing hydrocolloid impression materials can significantly decrease the load of pathogens such as *S. aureus* (15). The major limitation of this *in vitro* study was that the incorporation of Ag-NPs into irreversible hydrocolloid impression materials might affect their characteristics, depending on the type of irreversible hydrocolloid impression materials. Furthermore, complete assessments of the effects of Ag-NP size on antibacterial activities and characteristics of the irreversible hydrocolloids are necessary.

The major goal of this study was to design an appropriate static experiment to optimize the efficacy process of Ag-NPs against three microorganisms of *E. coli*, *S. mutans* and *C. albicans.* In this suggested treatment protocol, susceptibility of the highlighted microorganisms to irreversible hydrocolloids containing Ag-NPs generally depended on the microorganism species. Technically, BBD is one of the most often used techniques in optimization procedures (18). To the best of the authors' knowledge, BBD was used for the first time in this study to assess and optimize antibacterial factors of Ag-NPs, including Ag-NP concentration, powder-to-water ratio and mixing time, The BBD is an independent quadratic design that excludes embedded factorial and fractional factorial designs. Treatment combinations are located at the midpoints of the process space edges and in the center in this design (18). To fit the model, it is necessary to investigate statistical parameters. Correlation coefficient (R^2_{Sqared}) is a measure of the fitting of the predicted model with data resulted from the experiments. The R^2_{Adj} or the corrected correlation coefficient, similar to R^2_{Sqared}, is an indicator for showing usefulness of the model with more accuracy because it represents degrees of freedom (16). In this study, high correlation coefficients (0.9988 for Response 1,

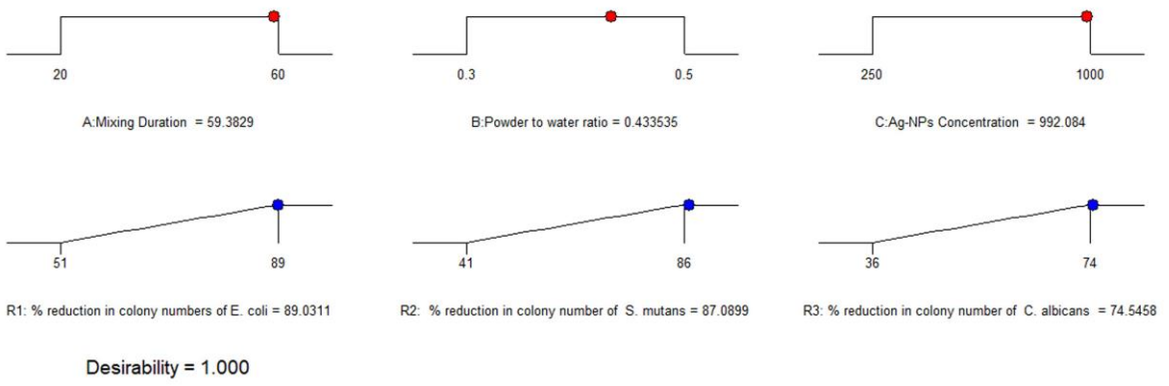

Fig. 4. Results for the optimization of independent variables

0.992 for Response 2 and 0.9988 for Response 3) revealed that the predicted model was well fitted to the data. The PRESS (predicted residual sum of squares) is predicted to assess extents; to which, the model is sensitive to its constructive data. The lower the parameter is, the stronger the model is (8.30). The R^2_{Pred} or the predicted correlation coefficient is a combination of the PRESS and R^2_{Sqared}. The more value is, the more powerful the model is. Adequate accuracy of the model is linked to signal-to-noise ratio of the model and the ratio should be greater than 4; similar to the current model. The adequate precision in the model was 15.546 that indicated a good signal-to-noise ratio. Furthermore, low standard deviation of the model (0.67) demonstrated high accuracy of the predicted model (18).

CONCLUSION

In the present study, antimicrobial process optimization of irreversible hydrocolloid impressions incorporated with Ag-NPs was carried out using RSM. The BBD model of RSM was highly precise in antimicrobial processes of the irreversible hydrocolloid impression materials. From the results of statistical design, disinfection efficiency dramatically increased with increasing of Ag-NP concentration, powder-to-water ratio and mixing time. Furthermore, the optimum conditions included mixing duration of 59.38 s, powder-to-water ratio of 0.400 and Ag-NP concentration of 992.0 ppm. However, further biological, mechanical and physical characteristics of the resulting impression materials should be investigated for a better verification of the protocol feasibility.

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