Journal of Hazardous Materials 180 (2010) 212–216

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Understanding the toxicity of aggregated zero valent copper nanoparticles against *Escherichia coli*

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ARTICLE INFO

Article history:
Received 24 October 2009
Received in revised form 30 March 2010
Accepted 7 April 2010
Available online 13 April 2010

Keywords:
Copper nanoparticles
Antibacterial activity
E. coli
Toxicity
Centroid design

ABSTRACT

Copper nanoparticles are used in wide variety of applications and in the current study we report the antimicrobial activity of these particles. Influence of pH, temperature, aeration rate, concentration of nanoparticles and concentration of bacteria on the toxicity of copper nanoparticles against *Escherichia coli* have been studied using a centroid mixture design of experiment. The linear and quadratic regression model shows that the toxicity of copper nanoparticles not only depends on the primary effect of the parameters tested (pH, temperature, aeration, concentration of *E. coli* and concentration of nanoparticles), but also on the interactive effect of these parameters.

1. Introduction

Manufactured nanoparticles are at the forefront of nanotechnology and are being used in a variety of applications. For example, zero valent copper nanoparticles (ZVCN) are extensively used in electronics, ceramics, films, polymers, inks, metallics, lubricant oil and coatings [1]. In addition, ZVCN have shown great promise in osteoporosis-treatment drugs, additives in livestock and poultry feed, antibacterial materials, and intrauterine contraceptive additives [2]. The increase in production and applications of ZVCN makes exposure to the natural environment of these compounds more likely. The release of nanoparticles into the environment can be either due to spills and use of products (direct emissions) or indirectly via landfills, waste incineration plants, and wastewater treatment facilities [3]. Numerous studies have established the toxic nature of the particles in animal and plant models. Studies have shown that when mice are exposed to ZVCN, severe impairment in the kidney, liver and spleen is observed [4]. Moreover, metabolic alkalosis and copper accumulation in the kidneys was detected in mice that were orally exposed to ZVCN [5]. The confirmation of the toxicity of ZVCN was provided by Lei et al. [6]. Lee et al. reported the phytotoxicity of the copper nanoparticles [7]. However, in contrast there is no detailed study carried out to investigate the toxicity of copper nanoparticles on microorganisms. Yoon et al. reported their preliminary studies on the toxicity of copper nanoparticles on *E. coli* and *Bacillus subtilis* using agar plate assay [8]. Their assay showed the antimicrobial characteristics of the particles on both the microbes tested. Esteban-Cubillo et al. have shown that copper/sepiolite nanoparticles strongly inhibit the growth of *E. coli* and *S. aureus* by 99.9% [9]. Ruparelia et al. showed the specificity of copper nanoparticles to selectively inhibit the growth of few strains of *E. coli* [10]. Recently, we reported that ZVCN influences the production of extracellular cellulose degrading enzymes in white-rot fungi *Trametes versicolor* [11]. In the current study, we report the dependence of the toxicity of ZVCN to *E. coli* on the concentration of nanoparticles, concentration of bacteria, pH, temperature, and aeration rate.

2. Experimental

2.1. Materials

All chemicals and reagents were of reagent grade and were stored according to the vendor's instructions and used as received. Nanoparticles were obtained from Sun Innovations, USA. Copper nanoparticles were 99.8% pure (metal basis) with an average particle size of 25 nm (the range of diameter is 2–60 nm). The

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doi:10.1016/j.jhazmat.2010.04.016

Table 1
Simplex centroid design for understanding the toxicity of copper nanoparticles on *E. coli* and the observed toxicity in each condition.

Run no.	pH x_1	Temp x_2 (°C)	Aeration x_3 (rpm)	[Nano] x_4 (ppm)	[*E. coli*] x_5, % (v/v)[a]	Toxicity %
1	6.80	35.6	160	500	4.10	61
2	8.00	33.5	100	312.5	2.60	17
3	6.50	33.5	400	312.5	2.60	6
4	6.50	44.0	100	312.5	2.60	8
5	6.50	33.5	100	1250	2.60	3
6	6.50	33.5	100	312.5	10.10	7
7	6.00	37.0	200	625	5.10	79
8	7.00	37.0	0	625	5.10	44
9	7.00	30.0	200	625	5.10	55
10	7.00	37.0	200	0.00	5.10	22
11	7.00	37.0	200	625	0.10	−52
V1	6.63	300	34.4	391	3.23	53
V2	6.63	125	40.5	391	3.23	52
V3	6.63	125	34.4	938	3.23	60

[a] The flasks having *E. coli* dilution of 0.1 and 10.1 have the cell load of 3.2×10^4 and 6.37×10^6 cells/mL, respectively.

nanoparticles are spherical with surface areas of 30–50 m^2/g (information received from the vendor).

2.2. Toxicity studies

To study the influence of nanoparticles on *E. coli*, the culture was grown in nutrient broth medium overnight at 30 °C, 200 rpm. Nutrient broth was prepared in 100 mM phosphate buffer with the pH under study and then diluted to a 5 mM concentration. *E. coli* was diluted as described in Tables 1 and 2, and 1 mL of the inoculum were added to 49 mL of nutrient medium in 125 mL Erlenmeyer flask containing ZVCN. The nanoparticles were added to the flask right before the addition of *E. coli*. The flasks were incubated for 30 min at different temperatures and aeration rate. Serial dilution and plating was carried out at the end of the experiment and agar plates incubated at 30 °C for 24 h to determine the colony forming units (CFUs). The percent toxicity of nanoparticles was determined by comparing the number of CFU present in the media after the incubation as compared to the number of CFU at time zero. A negative toxicity value in Tables 1 and 2 indicate that the number of CFU at the end of the experiment was higher than at time 0.

Most of the toxicity studies carried out on nanoparticles stabilize the particles using surfactants, polysaccharides or other stabilizing agents. The stabilizing agents prevent the aggregation of nanoparticles and allow the measurement of toxicity of pure nanoparticles. In such studies, the toxicity is measured as the relative toxicity of the nanoparticles as compared to the control experiments carried out using only the stabilizing agent. However, in nature it is less likely that the nanoparticles will be stabilized once the nanoparticles are released and they would form aggregates of various sizes. The eventual toxicity of the nanoparticles will be because of the combined effect of all the particle aggregates. To better mimic the natural conditions, ZVCN were added in the powder form in this study and not as stabilized particles. The organic rich environment present in nature is simulated by the presence of organic media ingredients in the nutrition broth.

2.3. Dynamic light scattering

Dynamic light scattering (DLS) was used to measure the rate of ZVCN aggregation at various temperatures and pH. Particle size measurements were carried out using a light scattering technique from Zeta Sizer Nano (Malvern Instruments). The instrument uses a 633 nm wavelength laser to measure the size distribution of suspended particles. ZVCN were dispersed in 10 mM phosphate buffer of various pH to obtain 100 ppm concentration which upon ultrasonication for 5 min was used for size measurement. Also, the size of the particles was measured again after aging for 30 min. The particle sizes are reported based on the intensity as well as the volume distribution.

2.4. Statistical analysis

All experiments were performed in duplicates and the results presented are the average of the duplicate experiments. Prior to regression analysis, any negative toxicity values were converted to zero. The regression analysis of the data was carried out using Microsoft Excel® 2007 and the ternary plots were obtained using Statistica software 8.0 (Tulsa, USA).

3. Results and discussion

Five environmental parameters (hereafter called independent variables) that are presumed to be play a significant role in the toxicity of the nanoparticles (hereafter called dependent variable) against the bacteria were selected. The five independent variables were pH, temperature, aeration rate, concentration of nanoparticles and the concentration of bacteria. The influence of these independent variables on the toxicity of ZVCN was investigated using a centroid mixture design of experiment. Centroid screening mixture design is an experimental design that was recently proposed as an alternative to traditional design of experiments [12]. This design helps understand the influence of independent variables on the dependent variable by taking into consideration the primary and the interactive influence, while reducing the number of experiments that must be performed [12]. The centroid mixture design for the study of ZVCN toxicity towards *E. coli* using the five independent variables is shown in Table 1. The range of pH, temperature, aeration and concentration of the bacteria being tested were selected based on the most likely natural conditions that could be found in an aquatic environment. The range for the concentration of nanoparticles was determined by the smallest possible amount of the nanoparticles that could be weighed accurately under laboratory conditions.

Aggregation of nanoparticles has been shown to have significant toxicological implications [13]. DLS was used to understand the aggregation of ZVCN as a function of pH and temperature. Table 3 illustrates the average size and percent volume of the aggregates measured at time 0, and after 30 min of incubation. An incubation period of 30 min was chosen for the DLS study because of a similar incubation period used in toxicity experiments. When the ZVCN were added to the pH 6 buffer at 30 °C, most of the nanoparticles aggregated into particles measuring 600 nm after 30 min of incubation with only 2% of the fraction in the microparticle range. In

Table 2
Placket–Burman design for understanding the toxicity of copper nanoparticles on *E. coli* and the observed toxicity in each condition.

Run no.	pH x_1	Temp x_2 (°C)	Aeration x_3 (rpm)	[Nano] x_4 (ppm)	[*E. coli*] x_5, % (v/v)[a]	Toxicity (%)
1	6	30	0	0	10.1	−14
2	8	30	0	0	0.1	−35
3	6	44	0	0	0.1	−17
4	8	44	0	0	10.1	15
5	6	30	400	0	0.1	−13
6	8	30	400	0	10.1	17
7	6	44	400	0	10.1	−3
8	8	44	400	0	0.1	−7
9	6	30	0	1250	0.1	15
10	8	30	0	1250	10.1	20
11	6	44	0	1250	10.1	91
12	8	44	0	1250	0.1	25
13	6	30	400	1250	10.1	74
14	8	30	400	1250	0.1	0
15	6	44	400	1250	0.1	100
16	8	44	400	1250	10.1	100

[a] The flasks having *E. coli* dilution of 0.1 and 10.1 have the cell load of 5.04×10^4 and 6.54×10^6 cells/mL, respectively.

Table 3
The size and vol% of the copper nanoparticles aggregates under different pH and temperature.

pH	Time (min)	Temperature (°C)	Size 1 (nm)	Vol%	Size 2 (nm)	Vol%	Size 3 (nm)	Vol%
6	0	30	142.18	16	672	34	5202	50
	30				600	98	4832	2
7	0	30	343	43	2587	25	4993	32
	30		388	31	2312	28	5270	41
7	0	40	162	25	470	45	5482	30
	30		373	15	679	32	4280	52
8	0	30	651	30	4702	37	5331	33
	30		827	46	996	11	5364	43
8	0	40	181	38	323	25	4688	36
	30		228	40	1618	51	5590	8

contrast, at pH 8 under same temperature, there is a significant fraction of the nanoparticle aggregated into microparticles (59%). It can be inferred that as the pH increases in the chosen range, the fraction of nanoparticles in the nanometer range decreases. Agglomeration of the copper nanoparticles is expected to be higher close to the iso-electric point (pH 7.5–8.5). As the pH decreases, the agglomeration also decreases which is supported by the data. The DLS results also show that increasing the temperature has the opposite effect to that of pH (see Table 3). The average aggregate size decreases as the temperature increases.

When one compares the toxicity values obtained in the first eleven experimental runs (Table 1, runs 1–11), all of the runs except the eleventh show some degree of toxicity. In the eleventh experiment, a 52% increase in the *E. coli* load was observed. The highest toxicity was found in the seventh run where the number of *E. coli* cells decreased by 79%. Linear and quadratic models were obtained using regression analysis. The linear model has an R^2 value of 0.57, and is given in Eq. (1):

$$toxicity\ (\%) = -4.2x_1 + 17.28x_2 + 9.6x_3 + 39.6x_4 + 74.7x_5 \qquad (1)$$

Based on the coefficients, when one ranks the variables from the variable having the greatest influence on toxicity to the least, we see that the concentration of *E. coli* (x_5) has the highest positive influence, while aeration (x_3) has the least positive influence. A positive influence would indicate that as the value for the variable increases, the observed toxicity increases. pH (x_1) has a negative coefficient indicating that with the increase in the pH of the environment, the observed toxicity decreases. The effect of pH and temperature (x_2) can be attributed by their influence on the aggregation of the copper nanoparticles. At lower pH and high temperature, the mean size of the aggregates is lower. One can formulate a similar hypothesis to understand the reason for the influence of aeration and *E. coli* concentration on the toxicity. At higher aeration, the large aggregates are constantly dissociated to lower size aggregates because of the shaking and shear, thereby increasing the toxicity of nanoparticles. *E. coli* has a large negatively charged capsule covering the cell. The interaction of the bacteria with nanoparticles can be assumed to decrease the aggregation and thereby increasing the observed toxicity of nanoparticles.

The toxicity of ZVCN against *E. coli* is also dependent on time. When the nanoparticles were incubated with the bacteria at pH 6.5, 400 rpm, 33.5 °C and nanoparticle concentration of 312.5 ppm, no toxicity was observed until 15 min (Fig. 1). 57% toxicity was observed in the next 5 min, reaching 96% toxicity at 30 min (Fig. 1). It may be hypothesized that the observed toxicity of nanoparticles against *E. coli* is due to the release of copper ions from the nanoparticles. Under acidic conditions and higher temperature, the

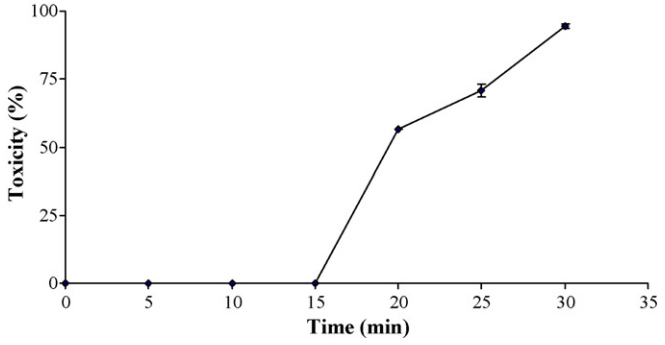

Fig. 1. Toxicity of ZVCN measured as a function of time at pH 6.5, 400 rpm, 33.5 °C and nanoparticles concentration of 312.5 ppm. The *E. coli* cell load was 1.4×10^5 cells/mL.

Table 4
Regression coefficients, standard error and the *P*-value obtained by analyzing the data described in Table 2 $R^2 = 0.99$.

Variable	Coefficient	*P*-value	Standard error
x_1	−127	0.15	87.31
x_2	−323	0.06	87.31
x_3	−267	0.07	87.31
x_4	−427	0.04	87.31
x_5	−493	0.04	87.31
$x_1 x x_2$	−3461	0.03	554.82
$x_1 x x_3$	1359	0.09	554.82
$x_1 x x_4$	4594	0.04	760.57
$x_1 x x_5$	0	–	0
$x_2 x x_3$	2070	0.06	554.82
$x_2 x x_4$	0	–	0
$x_2 x x_5$	5213	0.03	760.57
$x_3 x x_4$	0	–	0
$x_3 x x_5$	0	–	0
$x_4 x x_5$	0	–	0

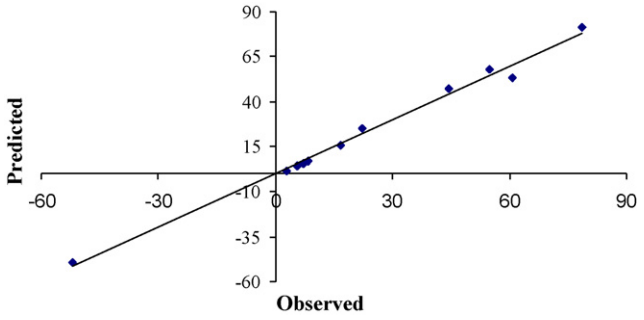

Fig. 2. *X*–*Y* scatter plot of the observed and predicted toxicity values along with the best-fit trend line. The *X*-axis represents the toxicity values described in Table 1 whereas *Y*-axis represents the predicted values based on the quadratic model described in Table 2.

mean size of nanoparticles aggregate is lower. This increases the surface area available for the solubilization of copper ions into the medium and hence the increase in the observed toxicity. During the initial 15 min of incubation, the concentration of ionic copper would not have reached the critical level required to show toxicity against the bacteria (Fig. 1). Ionic and metallic forms of copper have shown to be toxic to *E. coli* by generating hydroxyl radicals in the cytoplasm [14]. These radicals damage DNA, proteins and other molecules leading to cell death [15]. Indeed, further tests are warranted to investigate the mechanism of action for the toxicity of copper nanoparticles against *E. coli*.

A separate set of experiments was performed to confirm the influence of the independent variables on the toxicity of ZVCN (Table 2). The design of this experiment was based on a Placket–Burman design. A separate design was used to obtain a set of points in the design space different from those selected in the centroid design. A Placket–Burman design is known to be efficient in capturing primary effects of the independent variables [12]. In the experiments 1–8, no nanoparticles have been added and hence minimal toxicity is observed. These 8 experiments confirm that the extreme conditions of pH, temperature and aeration do not significantly affect the viability of the organism. When ZVCN is added to the medium (experiments 9–16, Table 2) the maximum toxicity of 100% is observed in experiments 15 and 16 only. Linear regression analysis of the results given in Table 2 gives the following model with a R^2 value of 0.69:

$$\text{toxicity } (\%) = -9.7x_1 + 1.48x_2 + 0.04x_3 + 0.04x_4 + 2.11x_5 \qquad (2)$$

Comparing Eqs. (1) and (2), one can see that in both the models pH has a negative coefficient, while other independent variables have positive coefficients. In addition, *E. coli* concentration is the most important variable affecting to the toxicity of ZVCN. Temperature (x_2) also has a high coefficient in the two linear models compared. While, nanoparticle concentration (x_4) has a low coefficient, the *P*-value of 0.002 indicates that that the variable plays a significant role towards the toxicity.

The low fit (lower R^2 value) of the linear models is an indication that there is high degree of interaction that is undetected by the linear model. The results given in Table 1 were used to obtain the quadratic model given in Table 4, which has a R^2 value of 0.99 with the standard error of regression of 10.5. A quadratic model for results described in Table 2 was not obtained because as stated earlier, a Placket–Burman design is highly appropriate for capturing primary effects and is not efficient in capturing secondary interactions [12]. Also, one needs to be cautious however when comparing the R^2 value in the linear model to that of the quadratic model. This is because when obtaining a quadratic model, the number of independent variables taken into consideration is higher, which provides a better fit. When the observed values for the eleven experiments used to develop the model and the predicted values obtained using the quadratic model are plotted against each other (Fig. 2). An equation of $y = 0.9919x + 0.1757$ provides a trend line that best fits the data. The constant of 0.1757 confirms the error within the model. Three-way and higher levels of interaction could also be presumed to influence the toxicity of copper nanoparticles. However, the three-way, four-way and five-way interactions have not been calculated in this study because a R^2 value of 0.99 for the quadratic model is sufficient. In addition, the number of experiments carried out in a centroid design of experiments is not enough to obtain an accurate higher order model.

Interestingly, the quadratic model shows that the primary effects of all the variables are negative and the observed overall effect for each variable is due to the interactions with other variables. The model shows that there is no two-way interaction for five of the ten possible two-way interactions. Confirmation of the interaction can be obtained from the ternary plots illustrated in Fig. 3(a–c). The plots clearly indicate the presence of interactions amongst the independent variables. When one compares the plots a to b, the region where maximum toxicity is predicted to be observed shifts away from the vertex of the concentration of nanoparticles (Fig. 3a) towards the concentration of *E. coli* (Fig. 3b). The difference in the two plots is that pH is held constant in (a) and temperature is held constant in (b). In Fig. 3c, very low toxicity is predicted when the aeration is high, while maximum toxicity is predicted towards the vertices of the other two variables. The shift in the zone of maximum toxicity confirms the interaction amongst the process variables. In all of the ternary plots, the two variables not shown on the plot were kept constant.

The *P*-values associated with model coefficients indicate the statistical significance for each variable, and confirms that interaction plays a significant role in the toxicity of ZVCN. A *P*-value of 0.05 or less indicates that the variable has a significant influence on the toxicity. Comparing the *P*-values of the quadratic model given in Table 4, the concentration of nanoparticles (x_4) and *E. coli* (x_5) are the only variables having a statistically significant primary influence. The interaction between pH (x_1) and nanoparticle concentration (x_4); pH (x_1) and RPM (x_2); and RPM and *E. coli* concentration are the only two-way interactions that could be considered having a significant influence on the toxicity of copper nanoparticles. Other two-way interactions are not statistically significant indicated by a *P*-value > 0.05. However, caution should be used in interpreting the *P*-values. These values could change if one uses a different design of experiment, or include higher level interaction in the model [16].

The validation of the quadratic model was performed experimentally by carrying out the three experiments denoted by V1–V3

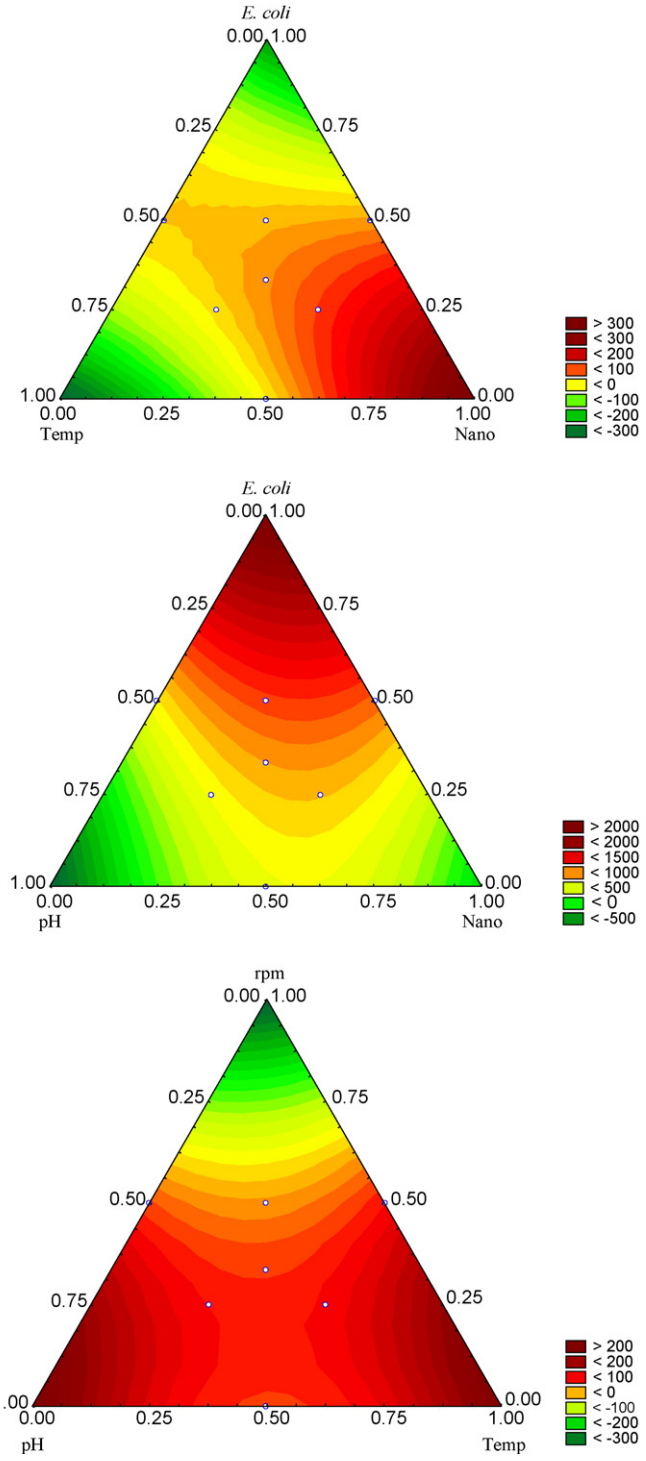

Fig. 3. Ternary plots of quadratic models predicting the toxicity under various conditions (the predicted values are represented in color scheme).

in Table 1. The quadratic model predicted the toxicity of 43%, 42% and 47%, respectively. All of the validation experiments were approximately 11% higher than the predicted value. When one considers the standard error of regression, the observed values are close to the expected values.

Thus, in conclusion the study shows that ZVCN will have highest toxicity on nanoparticles under acidic conditions and higher temperature, under high aeration and when the concentration of nanoparticles and bacteria are high. The toxicity of ZVCN changes significantly when any one of the independent variable is changed. The variables can be expected to affect the toxicity of other nanoparticles. Thus, one needs to be cautious when interpreting the toxicity of the nanoparticles when tested in one or few sets of experimental conditions.

Acknowledgement

The work was supported by National Science Foundation (Grant # CBET-714685) and Dowling College to VS.

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