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# Choosing safe dispersing media for $C_{60}$ fullerenes by using cytotoxicity tests on the bacterium *Escherichia coli*

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ABSTRACT

Assessment of $C_{60}$ nanotoxicity requires a variety of strategies for dispersing it into biological systems. Our objective was to determine organic solvent/surfactant combinations suitable for this purpose. We used *Escherichia coli* (ATCC# 25254) to determine the cytotoxicity of $C_{60}$ in solvents at concentrations up to 100 ppm. In this preliminary study we hypothesized that $C_{60}$ toxicity is directly correlated with its degree of dispersion in solution and that more solubilizing solvents induce higher toxicity. Test solvent concentration (1%) and Tween 80 (0.04%) were based on *E. coli* viability assay. Sonication was used to further enhance $C_{60}$ dispersal. The end-point response was measured with viability (in terms of $LC_{50}$) and general metabolic activity (in terms of $IC_{50}$) of *E. coli* cultures after exposure. The ultimate goal was to select safe dispersing media and enrich the database of $C_{60}$ nanotoxicity for NanoQuantitative-Structure–Activity-Relationship (NanoQSAR) applications. $LC_{50}$ range was 30 ppm to >400 ppm. $IC_{50}$ followed the trend. Among the six solvent combinations, DMSO combined with Tween 80 was the optimum combination for defining a dose–response relationship for assessing its toxicity to *E. coli*. However, *N,N*-dimethylformamide has the greatest potential to be a safe solvent for $C_{60}$ applications based upon its biocompatibility. Solvent solubility alone could not account for the cytotoxicity observed in this study.


## 1. Introduction

Research and development of engineered nanomaterials are steadily increasing but little is known about their environmental impact. $C_{60}$ fullerene and its derivatives have been shown to have various potential applications in organic photovoltaics, polymer electronics, anti-oxidants and biopharmaceuticals, and polymer additives [1]. According to a recent report by Nanotechnology Consumer Products Inventory in 2006 [2], carbon nanomaterials (including $C_{60}$) have the most dominant relative occurrence in consumer products currently on the market. As the nanotechnology industry develops further and demand increases, $C_{60}$ is expected to be produced and consumed in large quantities, which will eventually result in increased environmental exposure [2]. In aqueous systems, carbon nanoparticles can form aqueous suspended colloids and are subject to precipitation and aggregation because of their inherent hydrophobicity [3]. To evaluate the environmental impact of waterborne $C_{60}$ fullerene, its toxicity must be properly assessed. Hence, there is an urgent need to develop rapid, accurate, and efficient testing methods to assess these emerging materials with respect to environmental implications. Besides the significance of bacteria in biogeochemical cycling of chemical elements, the *in vitro* techniques with bacteria are preferred due to their rapidity and cost effective merits [4]. Derivatives of $C_{60}$ fullerenes could cause cellular damages by production of reactive oxygen species and inhibit the energy transduction process in microbial systems [5,6]. Hydrophobic nanomaterials can penetrate through tissue and pass into the lymphatics [7]. Genotoxicity of stable aqueous suspensions of colloidal $C_{60}$ fullerenes to human lymphocytes using the Comet assay, has been reported [8].

Conducting *in vitro* or *in vivo* toxicity assessment of $C_{60}$ fullerene requires enhanced procedures due to its hydrophobic nature. Therefore, the development of a special dispersion methodology must be addressed because most of the dispersing solvents are too toxic to the test biological systems [9]. Adding surfactants can promote the dispersion of hydrophobic substances in water. Consequently, that prospect was also explored in this study.

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**Table 1**
Cell viability count for organic solvents at different percentage concentrations. Organic solvent concentrations were tested using spreadplate technique at 4%, 2%, and 1%. Solubility of $C_{60}$ fullerene in different organic solvents[a].

| Organic solvents | Solubility ($\log S_{exp}$)[a] | %Cell viability (conc. 4%) | %Cell viability (conc. 2%) | %Cell viability (conc. 1%) |
|---|---|---|---|---|
| Methanol | −8.7 | 51.2 ± 2.3 | 97.9 ± 2.1 | 98.9 ± 1.2 |
| Ethanol | −7.1 | 70.1 ± 12.3 | 95.3 ± 12.1 | 95.8 ± 2.2 |
| Propanol | −6.4 | 98.1 ± 23.8 | 99.6 ± 15.1 | 99.7 ± 10.9 |
| DMSO | −6.0 | 33.8 ± 10.8 | 59.4 ± 11.3 | 89.9 ± 4.6 |
| *N*,*N*-Dimethylformamide | −5.3 | 47.0 ± 5.8 | 92.8 ± 5.8 | 124.0 ± 8.8 |
| Pyridine | −4.0 | 0 | 0 | 0 |

[a] From Liu et al. [16]. Solubility *S* is the mole fraction of $C_{60}$ in a particular solvent system. For convenience, the log of the mole fraction is used in this study to express solubility.

Tween 80 is a non-ionic surfactant and emulsifier that is derived from polyethoxylated sorbitan and oleic acid. It was used with the anticipation that it would further enhance the solubility of fullerene $C_{60}$ over the organic solvent working alone, sufficient to enable a meaningful assessment of $C_{60}$ cytotoxicity. Tween 80 was shown to enhance glucosyltransferase production in *Streptococcus mutans* [10]. As a biocatalyst, Tween 80 was also shown to increase biosulfurization in *Rhodococcus erythropolis* [11]. In enrichment suspensions of many bacteria cultures, Tween 80 is added at 0.04% as a part of the ingredients [12]. Therefore, in this study it was added in the aqueous solutions containing the study organic solvents with different concentrations of $C_{60}$ fullerene. The hypothesis is that cytotoxicity of $C_{60}$ fullerene increases with its solubility in the organic solvent. The study objectives were to select the appropriate organic solvents for assessing cytotoxicity of $C_{60}$ fullerene and choose the safe dispersing media for $C_{60}$ to minimize its environmental impact after release into the natural environments.

## 2. Materials and methods

### 2.1. Chemicals and instruments

All chemicals used in this study were of analytical grade. $C_{60}$ fullerene (99.5% purity) was purchased from Sigma–Aldrich (St. Louis, MO). LB nutrient agar, LB nutrient broth, and Petri dishes were purchased from Fisher Scientific (Houston, TX). Organic solvents including *N*,*N*-dimethylformanide, pyridine, methanol, ethanol, propanol, and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, Mo). Sodium chloride crystal for making physiological saline solution was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Bacterium *Escherichia coli* (ATCC# 25254) was purchased from the American Type Culture Collection (ATCC, Manassas, VA).

### 2.2. Measurement of viability with spreadplate counting

An *E. coli* suspension was introduced into LB nutrient broth and incubated at 37 °C overnight. The 18-h cultures were then centrifuged at 4000 rpm (3220 × *g*) for 10 min and resuspended in sterilized 0.8% physiological saline solution. Bacterial density was adjusted to $0.5 \times 10^9$ bacteria $ml^{-1}$ to $1.66 \times 10^9$ bacteria $ml^{-1}$ as determined by colony forming unit (CFU) counting on LB Petri dishes. $C_{60}$ fullerene was prepared with relevant organic solvents to make stock solutions at concentrations of 1000 ppm and 10,000 ppm. Stock solutions were sonicated (FS30 ultrasonic system; Fisher Scientific) for 1 h to break up any aggregates that might have formed in the organic solvent. Six groups were prepared: a control consisting of 0.8% saline, 1% organic solvent and 0.04% Tween 80, plus five treatments with $C_{60}$ added at concentrations of 1 ppm, 10 ppm, 25 ppm, 50 ppm, and 100 ppm respectively. Each $C_{60}$ fullerene stock solution was further sonicated for 10 min at 45 °C before being added to the bacterial culture solutions. Organic solvent concentration was 1% (see detailed description at the beginning of Section 3) in each sample vial containing *E. coli* in 2 ml of physiological saline solution (final concentration 0.8%). Solubility was monitored with UV–vis spectrophotometry (UV 160, Shimadzu; 340 nm) and further verified by the reproducibility of the toxicity data. The vials were then put into the shaker (Standard Multi-Tube Vortexer; VWR Scientific Products, Batavia, IL) for mixing at 25 °C for 2 h. After exposure, the samples were spread on LB nutrient agar Petri dishes using sterile techniques. The Petri dishes were then placed in an incubator at 37 °C for 24–48 h. The bacterial growth and viability were determined by CFU counting on the LB Petri dishes.

### 2.3. Assessment of general metabolic activity with $^{14}$C-glucose mineralization activity

Bacterial heterotrophic mineralization of glucose was measured as follows. After being washed 3 times with physiological saline, 100 μl of *E. coli* suspension was added to 10 ml of 0.8% saline. Controls included: kill control (autoclaved); control (saline buffer); control with 1% organic solvent; control with 0.04% Tween 80; control with 1% organic solvent and 0.04% Tween 80. Treatments consisted of $C_{60}$ fullerene introduced into 10 ml aqueous solutions containing 1% organic solvent and 0.04% Tween 80 to reach the final concentrations of 1 ppm, 10 ppm, 25 ppm, 50 ppm, and 100 ppm respectively. The controls and the experimental groups were then agitated for 2 h at a speed of 150 rpm. A mineralization count of $^{14}CO_2$ released during metabolic respiration of radio labeled UL-$^{14}$C D-glucose dissolved in ethanol (S.A. 2.48 mCi/mmol) was conducted after the 2-h incubation period. At time zero, the time of introduction, the pyrex milk dilution bottle was sealed with a silicone stopper attached with a center well containing a folded filter paper (Whatman #1) soaked with 0.7 ml of β-phenylethylamine for $CO_2$ trapping. They were allowed to sit overnight (12 h) after injection with 2N $H_2SO_4$ (to stop the mineralization reaction) following the 2 h incubation. Then, the filter papers were removed and placed in 20 ml scintillation vials containing 8 ml of Ultma Gold scintillation

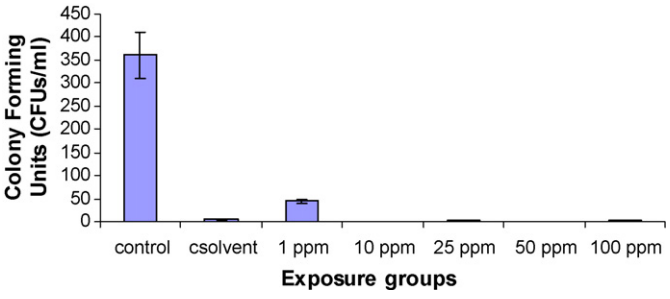


**Fig. 1.** $C_{60}$ fullerene suspended in 1% pyridine in physiological saline solution. Pyridine concentrations were 1 ppm, 10 ppm, 25 ppm, 50 ppm, and 100 ppm. Control consisted of physiological saline solution, and solvent is control with 1% solvent only.

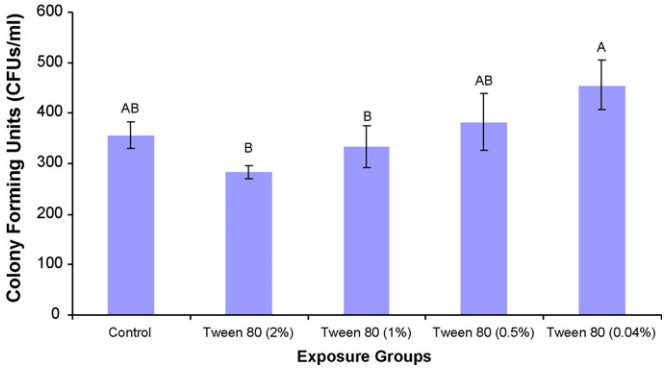


**Fig. 2.** Spreadplate analysis of *E. coli* bacteria after exposure to various concentrations of Tween 80. Means not sharing same label are significantly different at $p < 0.05$.

fluid from Packard (Meriden, CT, USA) and counted with a liquid scintillation analyzer (Packard Instrument, model TR 1600). Data was calculated from DPM (disintegrations per minute) readings to compute the percent mineralization [13].

### 2.4. Data and statistical analysis

Each experiment was conducted at least 2 times with triplicate samples of each treatment. Data of viability and metabolic activity measurements were used to compute $LC_{50}$ (concentration that causes 50% mortality compared to the control group with solvent and Tween 80) and $IC_{50}$ (concentration that causes 50% inhibition of metabolic activity compared to the control group with solvent and Tween 80) respectively by Probit regression [14]. The SAS System for Windows, V9.0 (SAS Institute, Gary, NC) was used for statistical evaluations. Differences among treatment and control groups were tested by one-way analysis of variance (ANOVA), followed by pairwise comparisons between groups using Duncan's test. Differences at $p < 0.05$ were considered significant. Histograms in the figures labeled with different letters represent significant differences.

## 3. Results and discussion

### 3.1. Selection of organic solvents

According to the instruction of the Comet assay reagent kit (Trevigen; catalog #4250-050-K), the prerequisite for conducting the genotoxicity test is that cell viability should be at least 75% viability or higher to rule out the false genotoxic effect caused by other mechanisms such as necrosis and apoptosis [15]. For cross-comparison with genotoxicity assessment in the future, therefore, we selected final candidate solvents that retained at least 75% of the viability of the saline control (Table 1). Toxicity of $C_{60}$ fullerene depends on its surface chemistry, particle sizes, and environmental conditions [16]. Solubility data of $C_{60}$ reported by Liu et al. [16] was adopted for initial consideration without challenge. Preliminary experiments of solubility vs. viability trials were conducted with 24 solvents. The list includes acetone, acrylonitrile, aniline, benzaldehyde, benzonitrile, bromoethane, butanol, butylamine, creosol, cyclohexane, 1,2-dichlorobenzene, ethanol, *N*,*N*-dimethylformamide, DMSO, methanol, N-methylaniline, nitroethane, pentanol, propanol, pyridine, quinoline, 1,2,3,4-tetrahydronap, thiophenol, and trichloroethylene. After the screening process, five were deemed relatively safe for use with *E. coli* and the results are summarized in Table 1. Other solvents severely inhibited bacterial viability at the concentration of 1%

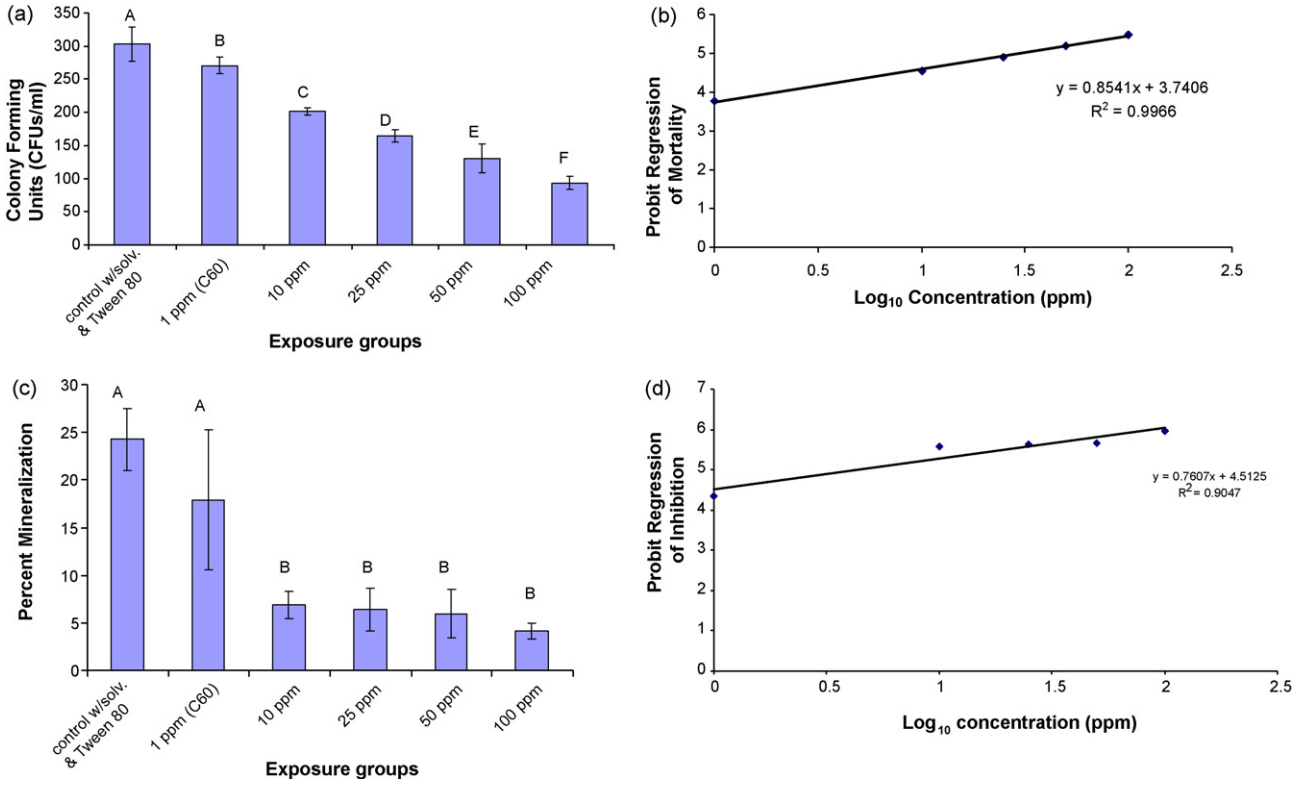


**Fig. 3.** (a) Spreadplate analysis of *E. coli* bacteria after exposure to 1% DMSO, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$. (b) Probit regression of spreadplate viability data on fullerene $C_{60}$ in 1% DMSO, and Tween 80. Calculated $LC_{50} = 30$ ppm. (c) $^{14}$C-glucose mineralization by *E. coli* bacteria after exposure to 1% DMSO, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$. (d) Probit regression of $^{14}$C-glucose mineralization data on fullerene $C_{60}$ in 1% DMSO, and Tween 80. Calculated $IC_{50} = 4$ ppm.

**Table 2**
Calculated $LC_{50}$ of $C_{60}$ fullerene suspended in 1% organic solvent with 0.04% Tween 80 (Probit regression from spreadplate viability data).

| Organic solvents | Solubility log $S_{exp}$ | Calculated $LC_{50}$ from spreadplates (ppm) | $R^2$ value |
|---|---|---|---|
| Methanol | −8.7 | 124 | 1.0000 |
| Ethanol | −7.1 | 55 | 0.5464 |
| Propanol | −6.4 | 484 | 0.7566 |
| DMSO | −6.0 | 30 | 0.9966 |
| *N*,*N*-Dimethylformamide | −5.3 | Indeterminable[a] | Not available |

[a] Because of the lack of dose-dependent response, $LC_{50}$ of *N*,*N*-dimethylformamide was indeterminable after the Probit regression. Consequently, $R^2$ value was not available.

(residual cell viability 0–42%). Among them, pyridine was included as a negative example for comparison. As indicated in Table 1 and Fig. 1 respectively, pyridine exposure led to complete elimination of bacterial viability. It was thus excluded from further experimentation.

Included in the list of solvents for final study were methanol, ethanol, propanol, *N*,*N*-dimethylformamide, and DMSO, each used at a concentration of 1% (v/v) in solution. These five chosen organic solvents showed a cell viability rates well above 75% as reflected in Table 1.

In medicine, alcoholic chemicals are used in applications as stabilizers and disinfectants. In addition, ethanol and related alcohols are routinely used in medical microbiology for *in vitro* testing as solvents [17]. The bactericidal activity of the alcoholic chemicals is due to mechanisms such as disruption of membrane structure or function [18], interference with cellular division, nutrient transport by membrane-bound ATPases [19], and variations in fatty acid composition and protein synthesis [20]. Dimethyl sulfoxide (DMSO), an amphipathic solvent, is frequently used to dissolve hydrophobic materials used in biological research. *N*,*N*-Dimethylformamide is used commercially as a solvent in vinyl resins, adhesives, and pesticide and epoxy formulations [21]. Among the study solvents, *N*,*N*-dimethylformamide appears to be the most bio-compatible. Specifically, it enhanced bacterial viability by 24% at 1% final concentration (Table 1). According to Farries et al. [22], the yield of enzyme-mediated peptide synthesis was improved by the presence of moderate concentrations (up to 5%) of *N*,*N*-dimethylformamide. We speculate that bacterial metabolism such as *in vivo* protein synthesis may be enhanced *via* this mechanism.

The $C_{60}$ solubilities in selected organic solvents, obtained from literature [16] and expressed as log $S_{exp}$, are shown in Table 1. Compared to the other solvents listed in Table 1, apparently $C_{60}$ has the highest solubility in pyridine and produces the highest mortality in *E. coli*. Therefore, our hypothesis is that cytotoxicity of $C_{60}$ fullerene increases with its solubility in the organic solvent.

### 3.2. Addition of Tween 80 to the solvents

In this study, Tween 80 was added to enhance dispersion of $C_{60}$ in the test aqueous solutions. According to the study concentrations reported in the literature [10–12], the effect of Tween 80 on bacterial viability was evaluated at the concentration, 0.04%, 0.5%, 1%, and 2% respectively. Because of the beneficial effect, concentration of 0.04% was chosen for all subsequent experimentations (Fig. 2). With the addition of Tween 80, aqueous solutions containing $C_{60}$ in various organic solvents at concentrations up to 100 ppm appeared to be homogenous and the reproducibility of the viability data was significantly improved over previous trials (data not shown). Moreover, adding Tween 80 to the saline control containing 1% organic solvent did not significantly affect bacterial viability of the control group with 1% organic solvent ($p < 0.05$; data not shown).

**Table 3**
Calculated $IC_{50}$ of $C_{60}$ fullerene suspended in 1% organic solvent with 0.04% Tween 80 (Probit regression from $^{14}$C-glucose mineralization data).

| Organic solvents | Solubility log $S_{exp}$ | Calculated $IC_{50}$ from $^{14}$C-glucose mineralization (ppm) | $R^2$ value |
|---|---|---|---|
| Methanol | −8.7 | 212 | 1.0000 |
| Ethanol | −7.1 | 56 | 0.9052 |
| Propanol | −6.4 | 160 | 0.3372 |
| DMSO | −6.0 | 4 | 0.9047 |
| *N*,*N*-Dimethylformamide | −5.3 | 491 | 0.7887 |

### 3.3. Cytotoxicity of $C_{60}$/organic solvent combinations

Among the five selected solvents, DMSO possesses the second highest capacity to solubilize $C_{60}$ (Table 1). Because of its antioxidant properties [23], we anticipated it to be a safe solvent for dispersing $C_{60}$ in this study. However, the observed toxicity data did not fulfill our expectation. First of all, it is the most inhibitive solvent on bacterial viability (Table 1) among the five finalists. The spreadplate viability and $^{14}$C-glucose mineralization methodologies both show it to promote $C_{60}$ highest toxicity/inhibition. The results are reflected in the dose–response charts and corresponding regression graphs (Fig. 3a and c). Based on Probit regression, the respective $LC_{50}$ and $IC_{50}$ values of viability and metabolic activity are 30 ppm and 4 ppm (Fig. 3b and d; Tables 2 and 3). Apparently the

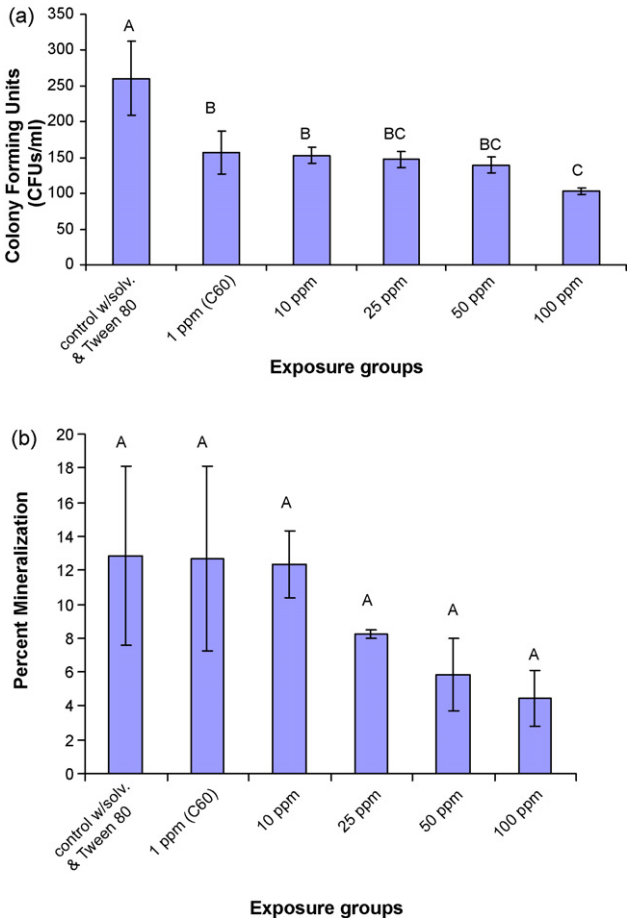


**Fig. 4.** (a) Spreadplate analysis of *E. coli* bacteria after exposure to 1% ethanol, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$. (b) $^{14}$C-glucose mineralization by *E. coli* bacteria after exposure to 1% ethanol, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$.

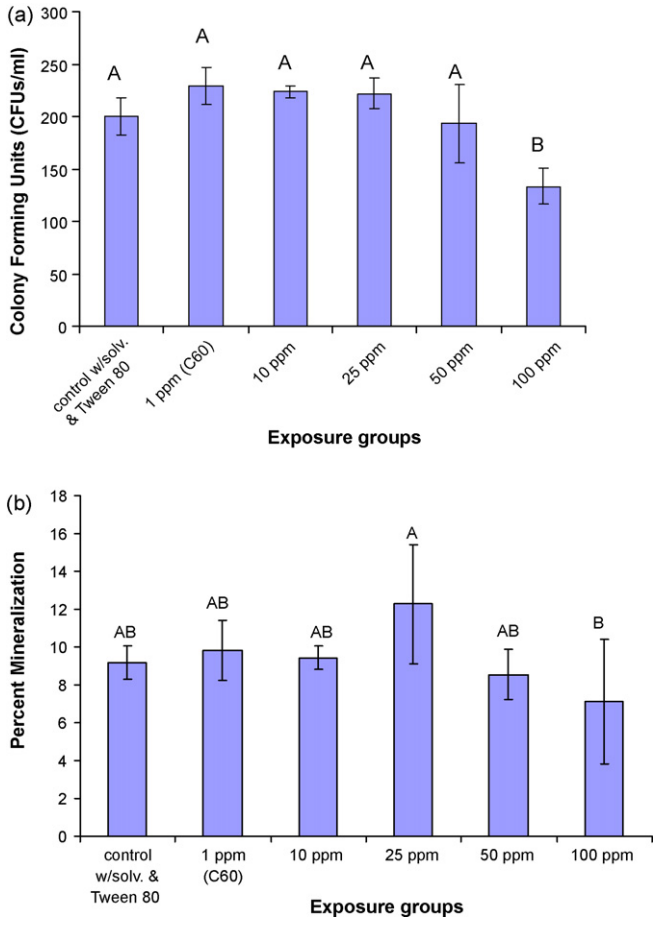


**Fig. 5.** (a) Spreadplate analysis of *E. coli* bacteria after exposure to 1% methanol, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$. (b) $^{14}$C-glucose mineralization by *E. coli* bacteria after exposure to 1% methanol, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$.

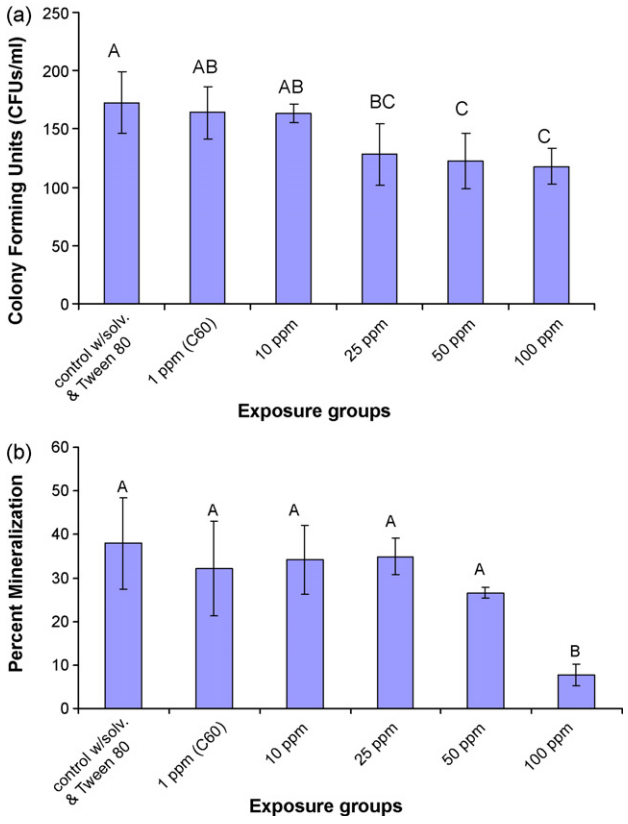


**Fig. 6.** (a) Spreadplate analysis of *E. coli* bacteria after exposure to 1% propanol, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$. (b) $^{14}$C-glucose mineralization by *E. coli* bacteria after exposure to 1% propanol, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$.

unique antimicrobial properties of DMSO make it useful in clinical applications such as treating urinary and dermatological disorders [24,25].

Fig. 4a shows the effect of ethanol on *E. coli* spreadplate viability and suggests a threshold of dose–response between 25 ppm and 100 ppm. Mineralization data are shown in Fig. 4b and is in general agreement with viability data in indicating a threshold between 25 ppm and 50 ppm. The calculated value of $LC_{50}$ for spreadplate viability is 55 ppm and the $IC_{50}$ for mineralization activity is 56 ppm (Tables 2 and 3). Among the five studied solvents, ethanol/$C_{60}$ should be the 4th most effective solvent for solubilizing $C_{60}$, based on the log $S_{exp}$ value in Table 2, but it is shown to be ranked second most toxic/inhibitive combination. This proved to be not the only inconsistency between solubility and toxicity in this study, opening the opportunity for further research to explain this phenomenon.

There is little evidence of a dose–response relationship of concentration and viability produced by methanol until 100 ppm as shown in Fig. 5a. Based on the Probit regression a $LC_{50}$ of 124 ppm is computed (Table 2). The results of mineralization for methanol, shown in Fig. 5b, also indicate a relatively flat response to increasing concentration of $C_{60}$. Like the viability count, there is the indication of a possible response threshold in the vicinity of 100 ppm. The calculated $IC_{50}$ is 212 ppm (Table 3).

For propanol, the $LC_{50}$ by spreadplate viability is an unexpectedly high 484 ppm, determined by the regression of the data graphed in Fig. 6a (Table 2). Mineralization data yielded a flat response past 50 ppm as seen in Fig. 6b, and an $IC_{50}$ 160 ppm computed by Probit regression (Table 3). Interestingly, simple linear regression yields an $IC_{50}$ of 64 ppm which is supported by the graph.

An enhancement in bacterial viability of 24% is seen after *E. coli* is exposed to 1% *N*,*N*-dimethylformamide (Table 1). As aforementioned in Section 3.1, we speculate this enhancement may result from the increase in bacterial anabolism after exposure to a moderate quantity of *N*,*N*-dimethylformamide. Fig. 7a shows *E. coli* exposed to $C_{60}$ in 1% *N*,*N*-dimethylformamide and 0.04% Tween 80. Microbial activity enhancement in every treatment as compared to the solvent control is evident. This may be due to the fact that bacteria used $C_{60}$ as a carbon source to increase the viability count. *N*,*N*-Dimethylformamide is readily degradable by microorganisms when in aquatic or in soil environments. Dimethylformamide does not induce gene mutation in any strain of *S. typhimurium* or in *E. coli* WP2uvrA and does not induce differential toxicity indicative of DNA damage in bacteria [26].

Some account must also be made for the possible interactions among solvents, bacteria and $C_{60}$ which produced higher viability than the saline control. Interestingly, a glutathione-$C_{60}$ derivative was reported to have the capability to prevent oxidative stress-induced cell death without evident toxicity [27]. Therefore, we speculate that the glutathione-$C_{60}$ derivative was formed inside the bacterial cells and helped mitigate the extent of nanotoxicity therein over the range of $C_{60}$ concentrations used in this trial. $LC_{50}$ was indeterminable from results obtained from spreadplate viability within this dose range. Mineralization produced an $IC_{50}$ of 491 ppm (Fig. 7b; Table 3), which is not unreasonable given that spreadplate produced no value due to viability enhancement. This

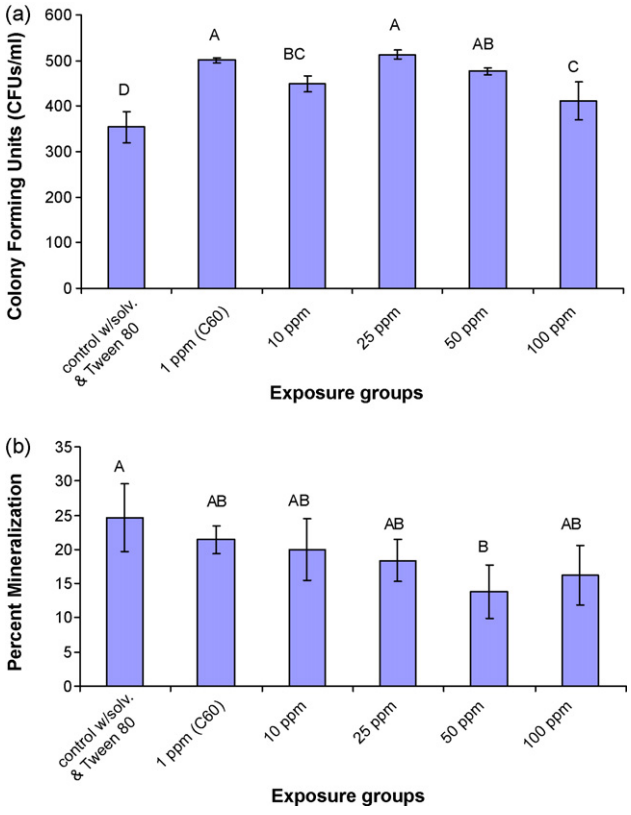


**Fig. 7.** (a) Spreadplate analysis of *E. coli* bacteria after exposure to 1% *N*,*N*-dimethylformamide, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$. (b) $^{14}$C-glucose mineralization by *E. coli* bacteria after exposure to 1% *N*,*N*-dimethylformamide, 0.04% Tween 80, and fullerene $C_{60}$. Means not sharing same label are significantly different at $p < 0.05$.

implies that if $LC_{50}$ existed, it would be well beyond the range of the concentrations used here.

As indicated in Section 1, derivatives of $C_{60}$ fullerene could cause cellular damages by production of reactive oxygen species and inhibit the energy transduction process in microbial systems. Therefore, bacterial viability and general metabolic activity were selected as the end-point responses in measurement of $C_{60}$ cytotoxicity. One additional advantage of using $^{14}$C-glucose for measuring bacterial mineralization activity is the ultra-sensitivity of the radiotracer technique. Except for the indeterminable $LC_{50}$ of spreadplate viability for *N*,*N*-dimethylformamide exposure, the relative toxicity/inhibition ranking of solvent/$C_{60}$ combination was similar in viability (Table 2) and metabolic activity (Table 3). Indeed, as that reported in the recent reports on the nanotoxicity research [28,29], the bacterium *E. coli* was conveniently utilized as a bioassay for evaluating $C_{60}$/organic solvent systems in this study. One caution to note is that bacterial heterotrophic activity of glucose is susceptible to both toxicity inhibitions and the competitions for bacterial utilization between glucose and the test substrates/contaminants [13].

Both spreadplate viability and $^{14}$C-glucose mineralization experiments could also suggest possible unexpected interactions taking place in the reaction mixtures. There may be autonomous enhancement of bacterial viability not only by the solvent (in some cases), and the Tween 80, but also by competitive substrate inhibition between glucose and the $C_{60}$ (e.g., Fig. 7b). This has vital implications of the need for branching research to sanitize the results of $C_{60}$ toxic effects on *E. coli*. Also, theoretically, a higher concentration of fullerene $C_{60}$ may need to be added to sample exposure groups to determine $LC_{50}$ by interpolation in preference to extrapolation. Ultimately however, practicality may take priority over this need because biocompatibility is an important criterion in selecting the dispersal media for comparative study of $C_{60}$ toxicity.

Increasing solvent solubilizing capabilities (Table 1) did not produce the expected corresponding increase in $C_{60}$ cytotoxicty as determined by inference from the values of $LC_{50}$ and $IC_{50}$. There is no correlation (work not shown) between these two variables for fitting all of the test solvents in this study. However, a fair comparison could also be made for solvents of similar structure. Within the group of aliphatic alcohols, a reasonable correlation could exist if ethanol is excluded ($R^2 = 0.54$; Table 2) and more alcohols are studied. The reported results here serve as a preliminary study. More specific studies are necessary to confirm or disprove the hypothesis.

## 4. Conclusions

In conclusion, our hypothesis was not substantiated; but interestingly we proved that there are factors other than solubility that determine the toxicity of $C_{60}$. Dimethyl sulfoxide (DMSO) combined with Tween 80 is the optimum combination for defining a dose–response relationship for assessing its toxicity to *E. coli*. In terms of potential health impact, *N*,*N*-dimethylformamide appears to be the safest among the solvents tested in this study for solubilizing $C_{60}$.

Conducting research into the cytotoxicity of hydrophobic nanomaterials requires some special approach (e.g., sonication of stock solution before adding to test solution) to achieve the dispersal of the test materials. Tween 80, the non-ionic surfactant used in this study, may interact with certain parts of the fullerene molecule making it more accessible to be metabolized when in the presence of an organic solvent.

Clearly, however, besides bacterial response to nanomaterials, consideration must be given to the interactions among solvents, Tween 80, and nanomaterials. Such interactions likely do occur and might lead to misinterpretation of the experimental results. For example, in their $C_{60}$ toxicity study with larval zebrafish, Henry et al. [2] reported that the observed toxicity was mainly caused by the decomposition products of the dispersal vehicle—tetrahydrofuran, instead of $C_{60}$ itself. Further study of these interactions in the house of chemistry is needed to disambiguate the findings.

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