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Treating Cell Culture Media with UV Irradiation against Adventitious Agents: Minimal Impact on CHO Performance

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Sterility of cell culture media is an important concern in biotherapeutic processing. In large scale biotherapeutic production, a unit contamination of cell culture media can have costly effects. Ultraviolet (UV) irradiation is a sterilization method effective against bacteria and viruses while being non-thermal and non-adulterating in its mechanism of action. This makes UV irradiation attractive for use in sterilization of cell culture media. The objective of this study was to evaluate the effect of UV irradiation of cell culture media in terms of chemical composition and the ability to grow cell cultures in the treated media. The results showed that UV irradiation of commercial cell culture media at relevant disinfection doses impacted the chemical composition of the media with respect to several carboxylic acids, and to a minimal extent, amino acids. The cumulative effect of these changes, however, did not negatively influence the ability to culture Chinese Hamster Ovary cells, as evaluated by cell viability, growth rate, and protein titer measurements in simple batch growth compared with the same cells cultured in control media exposed to visible light. © 2014 American Institute of Chemical Engineers Biotechnol. Prog., 30:1190–1195, 2014
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Introduction

Sterility is an ongoing challenge for commercial bioprocesses, particularly in the production of biotherapeutics, where sterility assurance levels are mandated across entire manufacturing processes. Major pharmaceutical companies have suffered significant losses due to both bacterial and viral contaminations at various stages of production.[1–3] Such incidents call attention to the need to address the risk profile associated with biopharmaceutical products. As an ingredient in the production process, culture media is a potential source of contamination that can propagate downstream and ultimately affect patient safety. Complementary media sterilization methods can confer additional safeguards for biopharmaceutical production and act as a viral barrier to mitigate contamination risks.

Sterility assurance levels of 6, or a probability of 10^{-6} that a single viable microorganism survives the sterilization process and is present in the final product, is the level of sterility that has become the standard requirement for pharmaceutical products and sterilization processes.[4] This level of sterility is traditionally accomplished via terminal sterilization processes by high heat and pressure,[5,6] which can be inappropriate for solutions having heat-labile components. High-temperature/short-time (HTST) pasteurization is also widely used as an upstream viral barrier. While this technology has been shown to be effective in inactivating viruses in media,[7,8] HTST has been shown to be incompatible with certain serum containing media.[8] Applying HTST can also cause phosphate- and calcium-based precipitates to form, ultimately impacting the operation of the HTST system.[9,10]

Alternative to heat treatments, aseptic procedures require all production equipment and components to be either "single-use" disposable or steam-sterilized before use. Cell media are often subjected to sterile filtration with 0.22 μm filters following aseptic steps to further preclude media contamination.[5]

Ultraviolet (UV) radiation is known to be an effective sterilant against various biological contaminants, including bacteria and viruses.[11–14] For example, active cytomegalovirus (CMV), can be reduced by one log with a UV fluence of 5 mJ/cm²,[15] while minute virus of mice (MVM) requires only 2 mJ/cm².[16,17] More resistant viruses, such as adenovirus require up to 306 mJ/cm² to achieve a log inactivation of approximately 6.[15,16,37]

UV disinfection is favourable for its non-thermal and non-adulterating characteristics, and has been adopted by several industries, including the biopharmaceutical industry, for packaging, and surface sterilization applications.[18–21] UV irradiation is able to approach the sterilization criteria of 6-log reduction with MS2 bacteriophage, which is used as a model challenge organism and used to assess reduction

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equivalent fluence of a UV system.[22–24] This makes UV irradiation a sterilization process fitting for diverse biopharmaceutical applications, such as in the sterilization of polyethylene bottles as well as the disinfection of air, water, and surfaces.[12]

The application of UV irradiation to cell culture media as a sterilization method has been limited thus far and conducted primarily in industry. Previous study on UV irradiation of media has suggested that treatment can result in changes to substrate concentrations or the production of various by-products,[25] possibly leading to cell growth failure[26] or changes in critical quality attributes (CQA) of the final product. The objective of this study was to investigate the effect of UV irradiation on the chemical composition of cell culture media and to determine the level of irradiation that can be tolerated before negative effects are observed in simple batch growth cell culture.

Materials and Methods

Experimental design

Two culture media were evaluated in this study: a protein-free, serum-free, chemically defined medium optimized for the growth of Chinese hamster ovary (CHO) cells and expression of recombinant proteins in suspension culture, as well as Media 199 (M199), formulation 11150, a fully defined protein-free, serum-free media used to culture chick embryo fibroblasts. Four UV irradiation doses were considered in this study, 110, 180, 250, and 400 mJ/cm^2. Due to the wide range of UV fluence at which bacteria and viruses are inactivated, these high-level dosages were selected to be effective against both bacteria, which are more UV-sensitive, and viruses, which tend to be more UV-resistant.[27] Fluence was estimated based on the log-reduction of MS2 bacteriophage in the media given that 110 mJ/cm^2 yields a 5-log reduction of this organism.

To best characterize the overall chemical changes in media, we used a nuclear magnetic resonance (NMR)-based metabolomics approach to characterize a large variety of media metabolites that could potentially be affected by the UV treatment. To achieve a high level of confidence in the observed effect of UV treatment on metabolite concentrations, each treatment was repeated three to six times per sample set, with multiple sample sets generated over a 4 month period. This allowed the separation of observation and process variability from the quantification of treatment effect.

The impact of UV irradiation on cell growth was determined by culturing cells in treated media. Two controls were used for the analysis: unmodified media that had not been treated in any way, which is referred to as samples receiving 0 mJ/cm^2, and non-irradiated samples that had been stirred and exposed to air for the same duration as the UV-irradiated samples.

Media irradiation

Irradiation of media was conducted at Trojan Technologies (London, Ontario, Canada). Irradiations were performed using a "Collimated Beam" device[28] incorporating a low-pressure mercury lamp emitting at 254 nm. This apparatus was designed to provide uniform, quantified irradiation to liquid samples, and the associated methods, including calibration, fluence determination, and quality assurance protocols, have been developed and standardized in the field of

water disinfection.[29] To enhance mixing, the irradiated volumes were reduced to 5 mL samples in 10 mL beakers, with continuous stirring. UV fluence values were calculated using the standard method based on measured UV irradiance and optical properties of the fluid. The UV dose values reported in this document were verified through disinfection testing as described below.

Verification of UV fluence

In order to establish the actual UV fluence values delivered to the culture media, a viral clearance test was conducted using a challenge organism inoculated into the culture media. The challenge organism, MS2, is a single-stranded RNA virus, with an icosahedral shell approximately 27 nm in diameter, used extensively in validation of UV disinfection systems for drinking water.[30]

MS2 bacteriophage was suspended in phosphate-buffered saline and irradiated in a collimated beam to various UV fluence values. The buffer used in this characterization had high transparency at 254 nm, typically 90% at 1 cm, so that the UV intensity gradient in the fluid was small, and therefore the uncertainty in the UV fluence was small. The 254 nm irradiance at the sample surface was measured using a radiometer with NIST-traceable calibration. The UV fluence gradient caused by optical absorbance of the fluid, along with the effects of beam divergence, non-uniformity, and surface reflection were all accounted for as recommended in the standard method. The samples were stirred during irradiation to ensure that all organisms were exposed to the same integrated UV fluence over the course of the irradiation. After irradiation, each sample was diluted serially and aliquoted into culture tubes containing 1 mL *Escherichia coli* broth culture and 20 mL of molten tryptone yeast extract glucose agar containing triphenyl tetrazolium chloride. The mixtures were mixed by inversion and plated into sterile Petri dishes. The agar was allowed to solidify and the plates were incubated at 35°C ± 0.5°C for 18–24 h before performing a plaque assay. The plates were then evaluated to determine the number of plaque forming units. By comparing with the control (non-irradiated) sample, the relationship between UV dose and the log-reduction of this population of MS2 was established.

With the UV-sensitivity of the organism determined, the UV dose applied to the culture media was determined by using MS2 as the dose indicator. This is sometimes denoted as "Reduction Equivalent Dose (Fluence)," since it is inferred from the log reduction of a well-characterized challenge organism. MS2 from the characterized population was inoculated into samples of the culture media, and the optical properties of the inoculated media were measured. The optical properties of the media were used to calculate the irradiation times necessary to achieve a desired UV dose in the collimated beam apparatus. The samples were then irradiated for the prescribed time, and then serially diluted and cultured as described above. The log reduction in numbers of active MS2 were used to calculate the UV dose received by the media in each irradiation by using the sensitivity of the MS2 as established by the buffer tests. The resulting Reduction Equivalent Dose vs. target dose has been plotted and may be seen in Figure 1. This relationship was used to determine the UV fluence values used in subsequent irradiations of mammalian cell culture media.
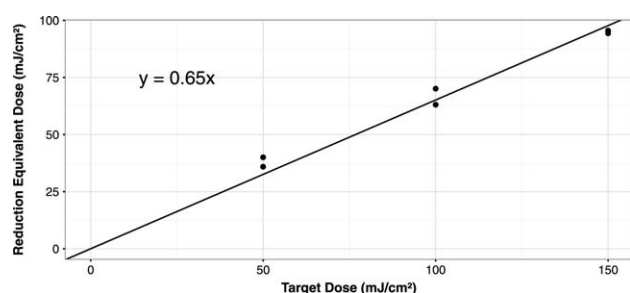
Figure 1. **Received UV fluence in commercial media measured by bioassay (MS2 bacteriophage) vs. target value. The "Reduction Equivalent Dose (R.E.D.)" applied to the culture media was determined by well-characterized MS2 phage as the dose indicator (Target dose).**

Cell culture

CHO[BRI] cells, obtained from the National Research Council Canada, were suspension-adapted cells grown in proprietary, serum-free growth media blend, BioGro-CHO, supplied by BioGro Technologies Inc. (Manitoba, Canada). The cells were adapted to grow in a commercial serum-free, chemically defined CHO media, supplemented with GlutaMAX[TM] (Invitrogen Corp., Burlington, Canada) and HT Supplement (Invitrogen Corp., Burlington, Canada). The parental culture was routinely maintained in shaker flasks kept in a humid incubator (37°C, 5% CO_2) and agitated at 100 rpm. Both the parental and experimental cultures were cultured in 125 mL, graduated non-pyrogenic polycarbonate Erlenmeyer flasks (Corning Inc., NY). Once the mother flask reached a viable cell density of $2–3 \times 10^6$ cells/mL, the experimental cultures were inoculated at a seeding density of 0.2×10^6 viable cells/mL.

For culture tests, the irradiated and control samples of CHO media were supplemented with GlutaMAX[TM] and HT Supplement after media irradiation and prior to inoculation. GlutaMAX[TM] and HT Supplement were not subjected to UV irradiation in these experiments. Multiple irradiated samples were pooled to achieve 15–20 mL volumes required for culture growth and supplemented as above.

Cell count

Each experimental culture was counted at 24-h intervals for 5 days using both the classic haemocytometer counting method, and with a Coulter Counter Z2 (Beckman-Coulter, Miami) for cell size distribution analysis. Samples counted in the haemocytometer were prepared in a 1:2 dilution with Trypan blue (10% w/v in PBS) before loading the diluted sample into the counting chamber. In circumstances where the cell count was greater than $3–4 \times 10^6$ viable cells/mL, the sample was diluted by a factor of 10 using CHO media as the diluent, while maintaining a 50:50 ratio of dye to sample and diluent. Cell size distributions were obtained from a Coulter Counter Z2 by loading samples diluted 100-fold in Isoton® II solution. Cell viability was also calculated daily based on haemocytometer counts.

NMR spectroscopy and metabolite profiling

NMR samples were prepared by the combination of 630 μL of media and 70 μL of internal standard composed of 99.9% D_2O with 5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid

(DSS) serving as a chemical shape indicator (CSI) and 0.2% w/v sodium azide to inhibit bacterial growth (Chenomx Inc., Edmonton, Canada). The solution was vortexed and pipetted into 5 mm NMR tubes (NE-UL5-7, New Era Enterprises Inc., Vineland, NJ) for scanning. NMR spectra were obtained with a 600 MHz Bruker Avance spectrometer, equipped with a Triple Resonance Probe (TXI 600). The spectra were acquired using the first increment of the NOESY pulse sequence with a 1-second presaturation pulse, followed by a 4-second acquisition time. All spectra processing was carried out with Chenomx NMR Suite 7.5 (Chenomx Inc., Edmonton, Canada). Baseline, phase, shim, and chemical shape corrections were all performed manually using tools available with the software. Briefly, baseline correction was carried out by the selection of cubic spline points to subtract from the observed spectra, while shim correction corresponded to reference deconvolution to remove line asymmetry.[31] Subsequently, compounds were quantified by targeted profiling. The observed spectra were superimposed with Chenomx's built-in library of chemical resonances, with metabolite concentration quantified using DSS as a reference compound (for more information on targeted profiling, see Weljie et al.[32]).

Antibody titer

After 5 days of culturing, the supernatant for the CHO cultures were collected. Each acquired sample was concentrated down to 1 mL volume using a Centriprep Centrifugal filter unit with an Ultracel-10 membrane (EMD Millipore, MA). The concentrated samples were passed through a Protein A HP Spin Trap column (GE Healthcare) and purified IgG antibodies were collected. Antibody titer (total extracellular protein) was determined using a Pierce[TM] BCA protein assay kit (Pierce Biotechnology, Rockford).

Results

NMR metabolite analysis

About 28 compounds were identified and quantified in the media by NMR, of which 12 compounds were found to have statistically significant trends with respect to UV irradiation: pyruvate, acetate, arginine, lysine, threonine, tryptophan, tyrosine, choline, methionine, formate, ethanolamine, and pyridoxine (in decreasing order of concentration change). When the concentration profiles of these compounds were compared with changes from exposure to visible light and air (without UV) in the control samples, it was found that five compounds had similar changes from visible light and air exposure: arginine, threonine, choline, methionine, and ethanolamine. These changes may be related to exposure to oxygen or to the effects of room light, but are not related to UV exposure, since there was negligible UV exposure in these controls. Concentrations of pyruvate, acetate, and formate were also observed to change with exposure to visible light and air, *albeit* much less than under UV treatment. Therefore, the observed concentration changes of only seven compounds could be directly linked to UV exposure—pyruvate, acetate, lysine, tryptophan, tyrosine, formate, and pyridoxine. The concentration profiles of these compounds were all approximately linear as a function of UV fluence (Figure 2). The specific concentration changes can be seen in Table 1, calculated per 100 mJ/cm^2 of UV fluence. Amino acids changed the least in relative concentration, ranging from decreases of
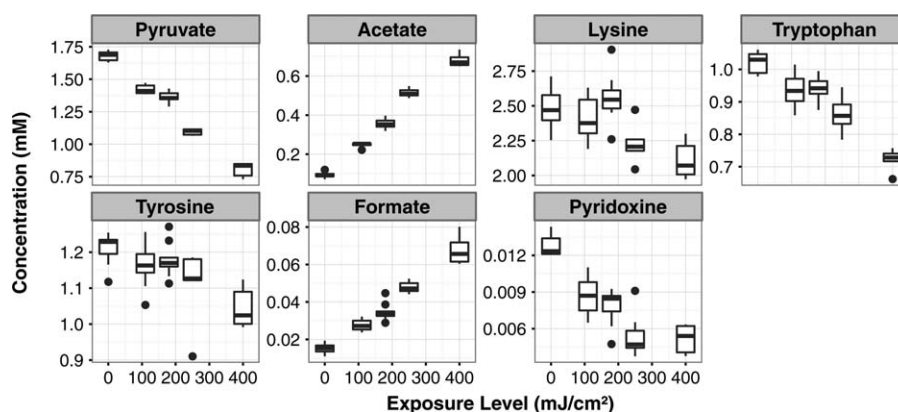
Figure 2. **Compound concentration as a function of UV fluence. The box-plots contain aggregated data from multiple experiments in which exposures were performed three to six times at each level.**

Table 1. Changes in Compound Concentration per 100 mJ/cm² of UV Dose*

Compound	Concentration Change (μM)	Concentration Change (%)
Pyruvate	-218.7 ± 6.6	-13.0 ± 0.4
Acetate	152.1 ± 3.4	164.0 ± 3.6
Lysine	-91.5 ± 21.4	-3.7 ± 0.9
Tryptophan	-70.6 ± 5.6	-6.9 ± 0.5
Tyrosine	-39.6 ± 7.2	-3.3 ± 0.6
Formate	13.2 ± 0.5	86.5 ± 3.5
Pyridoxine	-2.0 ± 0.2	-15.6 ± 1.6

*Linear regression was performed on the change in compound concentration as a function of UV dose with the presented number corresponding to the predicted concentration change per 100 mJ/cm² (with the $\pm$ representing standard deviation).

3%–7%. A much greater effect was observed for the carboxylic acids, with acetate concentration, for example, increasing twofold.

Cell culture

Cell culture performance was primarily assessed by viability, growth rate, and final cell concentration. Controls (exposed to visible light) were cultured alongside cultures grown in UV-treated media to account for changes in cell behaviour. Overall, UV-treatment of the media did not have a significant effect on cell growth. Therefore, any variability observed in cell growth and protein production can be attributed to random effects imparted by the experimental environment, such as visible light. Cell viabilities of both treated and control samples were routinely above 95%, with no significant difference between the two groups as tested by paired Student's *t*-test at the 95% confidence level. Additionally, no differences were observed in cell size distributions (data not shown). Growth rates can be seen in Figure 3a and the final cell concentrations in Figure 3b. Overall, the differences between treated and control cultures were not statistically significant.

In the course of this study, quail muscle fibrosarcoma (QM5) cells were also cultured in M199 media treated with a dose of 110 mJ/cm². No differences were observed between QM5 cells grown in UV-treated and those grown in control media. In both cases, the cells were able to reach confluence within 4 days, with no apparent changes in morphology. Higher UV doses were not assessed for this media.

Protein titer

Similar to the growth data, recombinant IgG protein titers from the CHO cells were found to be very similar for UV-treated and control media. Comparing the yield on a purely volumetric basis suggested that UV-treated media may result in somewhat better protein production (Figure 3c). However, when integrated viable cell density was used to calculate specific protein production levels on a per-cell basis,[33] there were no significant differences in protein yield (Figure 3d).

Discussion

Although UV irradiation has been previously reported to negatively impact cell growth,[26] the limiting UV dose values were not indicated. No negative effect was observed in this study for either CHO cells grown in UV-irradiated CHO medium or QM5 cells grown in UV-irradiated M199, suggesting that the impact of UV-irradiation cannot be generalized, or the effect is only encountered when pushing the culture beyond a simple single batch process. The metabolic changes observed due to UV irradiation of media did not impact the overall pH measurement of the media solutions used. It should be noted that we have observed pH shifts in other irradiated media (data not shown), though none in this study. This is in part due to the sodium bicarbonate buffer in the media and the relatively small absolute changes of impacted compounds, such as acetate.

Observed changes in substrate concentrations were also limited. Amino acid concentration changes that did occur were on the order of 1%–7% per 100 mJ/cm². Most cell culture media contain these compounds in excess so the extent of degradation of amino acids from UV irradiation, even at high UV doses, is unlikely to impact culture performance. The small changes in substrate concentration also meant that any specific degradation products were generally masked by overall compound variability, which we have previously found to be approximately 5%–10% for most compounds, measured as a coefficient of variation.[34] Pyridoxine was the only vitamin that could be observed with NMR, and therefore no general conclusions can be made about overall vitamin stability. However, the cell growth data indicates that the level of vitamin degradation was too low to impact cell growth compared with our control.

The largest changes in compound concentration in these media were observed for carboxylic acids. Pyruvate concentrations were found to decrease by approximately 13% per 100 mJ/cm², with acetate and formate increasing by 160%
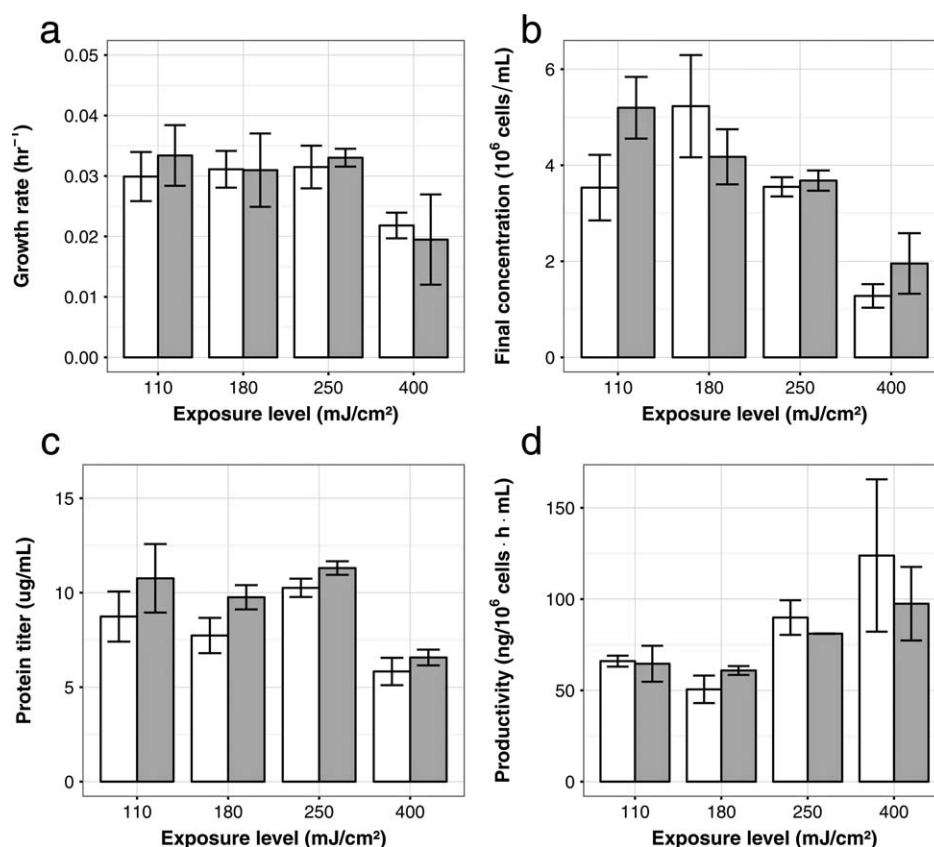
Figure 3. **(a) Comparison of cell culture performance on control (*white*) and treated (*gray*) media. In all cases, controls (exposed to visible light) were cultured alongside cultures grown in UV-treated media to account for changes in cell behaviour over a 4-month period. No statistically significant differences were observed between treated and control media for any of the culture parameters, as assessed with a two-sample *t*-test at a 95% confidence level. (a) Mean exponential phase growth rates (with standard errors) for cell cultures grown in UV-treated and control media. Values were calculated from the log-regression slope of concentration data from days 1–3. (b) Mean final cell concentrations (with standard errors) for cell cultures grown in UV-treated and control media. For the 250 mJ/cm^2 dose, the cultures grown on treated media resulted in nearly identical final concentrations, leading to overlapping error bars. (c) Mean protein yield (with standard errors) for cell cultures grown in UV-treated and control media. (d) Mean specific protein yield (with standard errors) for cell cultures grown in UV-treated and control media. Values were calculated by dividing the volumetric protein yield by the integrated viable cell density. For the 250 mJ/cm^2 dose, the cultures grown on treated media resulted in nearly identical specific protein yield, leading to overlapping error bars.**

and 90% per 100 mJ/cm^2, respectively. This corresponded to absolute changes of 218, 152, and 13 μM for pyruvate, acetate, and formate, respectively. The three compound concentrations showed parallel trends in both control (visible-light-exposed) and treated media. Considering the high degree of precision in its quantification (2% coefficient of variance including set-to-set variability), pyruvate concentration can be seen as an attractive marker for assessing the overall effect of UV treatment in cell culture media using NMR spectroscopy. As previously mentioned, no additional impact on cell growth was observed compared with the control in spite of such changes to these metabolites.

This decrease in pyruvate is not altogether surprising. Pyruvate has been previously identified to be a prominent antioxidant in biological systems and has been found to yield acetate when reacting with peroxynitrite, a powerful oxidant,[35] or hydrogen peroxide.[36] It may serve a similar role in the quenching of oxidative products formed during UV treatment. Based on absolute concentration changes, it would appear that acetate is the major product of such reactions, although formate may also be involved.

The UV doses applied in this study would achieve disinfection for most adventitious agents relevant to CHO cell

culture, such as mouse minute virus and reovirus.[3,11,27] Since filtration is seen as an effective method to remove larger virus and bacteria, UV disinfection is primarily seen as a complementary method to inactivate adventitious agents not captured by filtration.

Conclusion

The UV doses applied in this study did not functionally impair the culture media tested, as measured by cell growth and antibody production of CHO cells. Cell morphology was not impacted and the cell size distribution was comparable between cultures grown in UV-treated media and control media that had only been exposed to visible light. These findings were consistent at various fluence levels, even at very high exposure levels of 400 mJ/cm^2. QM5 cells were also unaffected by media irradiation, though media was only exposed to UV doses up to 110 mJ/cm^2. Changes to major media components were largely limited to carboxylic acids, with only minimal changes in amino acid concentrations. The findings in this study serve as a demonstration of some of the changes that can occur to cell culture media after applying UV sterilization. While these results cannot be generalized to

all cell lines and culture media, this study provides insight into the effects of UV-sterilization in cell culture applications. Further validation with investigations focusing on longer culture duration, higher cell densities, repeated passaging in UV-treated media, and ultimately the effect on protein product critical quality attributes is currently being undertaken.

Acknowledgments

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