

SyntheChol™ Synthetic Cholesterol for Cholesterol Dependent Cell Culture — Development of Non-Animal Derived Chemically Defined NS0 Medium

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Abstract

Traditional serum supplemented cell culture medium is fast becoming outdated as new technology permits the omission of most animal derived protein from the culture system. Cell lines derived from the NS0 myeloma cell line are rising in popularity for biopharmaceutical production, due to their high cell growth potential and their subsequent high production yields. However, because of their cholesterol auxotrophic nature, NS0 derived cell lines are particularly challenging for medium development. The first hurdle lies in the fact that there is no source of non-animal derived cholesterol. Second, cholesterol is not water-soluble and thus requires supplementation at the point of use. Both of these aspects are discouraging to those who need these cell lines for large-scale manufacturing. To address these issues, Sigma-Aldrich has produced SyntheChol™ (Product Code C1231); a synthetic, non-animal derived cholesterol. Initial results demonstrate that SyntheChol behaves similarly to animal-source cholesterol when dissolved in ethanol and supplemented in Hybridoma Medium, Animal Component Free (Product Code H4409). Furthermore, we have developed a sterile, liquid (500X), ready to add SyntheChol NS0 Supplement (Product Code S5442) with increased stability in cooler conditions. The results showed that SyntheChol NS0 Supplement is a stable and effective production-enhancing supplement as compared to both competitor supplements and natural cholesterol dissolved in ethanol. SyntheChol NS0 Supplement can be added to any medium prior to use. Finally, the development of the synthetic cholesterol, SyntheChol, has offered an opportunity of manufacturing an animal component-free, chemically defined NS0 medium.

Introduction

The NS0 cell line obtained from the European Collection of Cell Cultures (ECACC culture number 85110503) is described as a mouse myeloma cell line with lymphoblastic morphology. It is a subclone of the NS-1 cell line that is traditionally known to be cholesterol dependent. NS0 cells are cholesterol auxotrophs that are unable to manufacture cholesterol on their own. This condition is particularly challenging for a serum-free/protein-free medium manufacturer. NS0 derived subclones are quickly gaining ground in the biopharmaceutical-manufacturing arena as a cell line of choice for several reasons. First, NS0 cells are very hardy, proliferative cells. They grow in suspension and so have been observed under ideal conditions at densities of greater than 7 million cells per milliliter. Because they grow in suspension, they lend themselves easily to use in the larger and more complex stirred tank and perfusion bioreactor systems. Most importantly, NS0 recombinant clones are very productive.

Production rates can exceed 70 mg/L with minimal optimization work.

Cholesterol is not water-soluble. The lipid-like characteristics of cholesterol make it appear hazy in liquid medium. This condition worsens over time and is accelerated by storage in cooler conditions, which are required for most serum and protein-free media. Furthermore, until very recently there was no non-animal source of cholesterol on the market. So an "animal component free" medium was not possible. Recently Sigma-Aldrich Corporation developed a non-animal, synthetic cholesterol, called SyntheChol™ (Product Code C1231). Using this newly created SyntheChol, we have further developed a sterile, ready to add (500X) liquid, SyntheChol™ NS0 Supplement (Product Code S5442) with increased stability in cooler conditions. In this study, the effects of SyntheChol and SyntheChol NS0 Supplement on cell growth and rlgG production in NS0 cells and NS0-derived recombinant clones are presented.

Materials and Methods

Cell Lines and Cell Culture Media

Stock NS0 and other proprietary NS0 derived recombinant clone cell stocks were maintained in Sigma-Aldrich Hybridoma Medium, Animal Component Free (Product Code H4409), supplemented with 10 mM L-Glutamine (Product Code G7513), and 5 mg/L animal derived cholesterol (Product Code C3045 and/or C8667) or SyntheChol (Product Code C1231) which was dissolved in 200 proof ethanol at 16 mg/ml.

Cell Culture and Cell Growth Assays

Cell stocks were maintained at 37 °C, 5% CO₂ and 95% Rh in a Thermo Forma model 3310 incubator. Growth vessels were Corning 75 cm² flasks (Product Code C0427) during the initial days of culture and then switched to Techne spinner flasks (catalog number 6027689). Stock cultures were maintained for no more than 25 passages between 50,000 and 1,000,000 cells per milliliter and were passed three times per week.

For studies involving competitors' media and supplements, cell stocks were taken from serum containing basal medium and adapted to the respective test media. These multiple stocks were then used to inoculate simultaneously running conditions, so that the cells were pre-adapted to all conditions being run and no single condition had any advantage over another. All cell stocks were harvested with at least 90% viability and were in log phase growth.

Techne spinner flasks were set up with base medium and test conditions such that the volumes in the flasks were all equal. The spinner flasks were incubated for one hour prior to cell inoculation. The cell stocks were counted and viability was established to standardize cell passages. The remaining stock

cells were harvested by centrifugation and then resuspended in Sigma-Aldrich Hybridoma Medium, Animal Component Free with no cholesterol supplementation. A viable cell count was performed again. All assays were set up with the cell seeding density at 150,000 viable cell/ml. All assays included a no cholesterol control and a positive cholesterol (either sheep wool or SyntheChol where applicable) control. All reported results are the average of two replicate spinner flasks for each condition. Cell counts were done on a Scharfe Systems CASY-1 cell counter and viability was established on a hemocytometer by Trypan Blue dye exclusion method.

Quantitation of Recombinant IgG

Samples were centrifuged to remove cells at 1000 xG and supernatants were submitted for analysis. An affinity chromatography method was employed utilizing an analytical column designed for very rapid mass transport. The particle surface is coated with a cross-linked polymer, derived with recombinant protein G. The protein G has a high affinity for IgG under neutral conditions. The column does not retain other proteins such as albumin. After the unbound proteins have been washed from the column, the bound IgG is released by an acidic eluting solution. The amount of IgG in the subsequent peak is detected and quantitated by UV absorbance at 210 nm. This assay was performed using a Dionex model #AD 20 UV VIS detector with a Protein G cartridge from Applied Biosystems catalog number 2-1002-00.

Mass Spectrometry

Analytical comparison of animal-derived cholesterol (C8667) and SyntheChol (C1231), by reversed phase HPLC with UV and MS detection was performed using the following equipment and parameters: Waters 2690; Phenomenex C18 (2), 5 mm, 50 x 2 mm column; column temperature, 40 °C; isocratic mobile phase, 95% ACN 5% H₂O; flow 0.100 mL/min; injection volume, 10 µl; run time 20 min; Micromass LCT; ionization by positive ion atmospheric pressure chemical ionization (APCI).

Results and Discussions

As shown in Figure 1, SyntheChol performs as well as both animal-derived cholesterol and fetal bovine serum in supporting NS0 cells growth.

Comparison of Cell Growth with Animal-Derived and Synthetic Cholesterol (SyntheChol™)

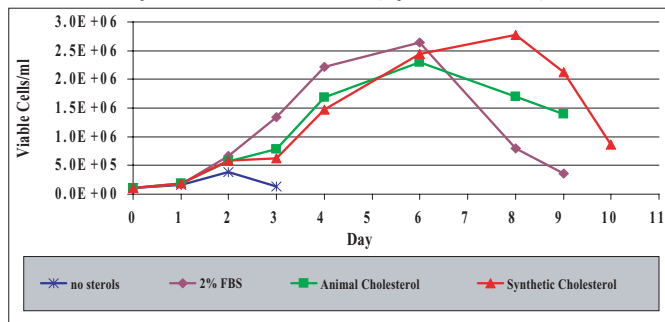


Figure 1. Comparison of animal-derived cholesterol and synthetic cholesterol (SyntheChol, Sigma C1231) in supporting NS0 cell growth. Cell growth of NS0 cells were tested with Sigma hybridoma medium (H4409) supplemented with 2% FBS, 5 mg/L of animal-derived cholesterol and synthetic cholesterol. Both animal-derived and synthetic cholesterol were dissolved in Ethanol. The synthetic cholesterol exhibited growth characteristics comparable to the animal-derived cholesterol and FBS.

Furthermore, analytical data shows that the physio-chemical behavior of SyntheChol is in agreement with that of animal derived cholesterol (Figure 2). The two products have similar chromatographic profiles as displayed in Figure 2 (a-d). Although purity was not an objective of the analysis, the UV chromatograms show that the samples are quite pure. The only major peak detected was that of cholesterol. The two products yielded nearly identical mass spectra (background subtracted) see Figure 2 (e-f). The only major ion detected was m/z 369.40. The monoisotopic molecular weight of cholesterol is 386.3549. Under the ionization conditions the C-O bond is extremely labile and the loss of the OH group results in a carbocation with the monoisotopic mass of 369.3521. This is in agreement with the observed peak in the mass spectra. The accurate mass measured for C8667 was 369.3555. The accurate mass measured for C1231 was 369.3548. These two masses are in agreement within 0.7 mDa. Therefore these accurate masses indicate that the ions have the same empirical formula. Both accurate mass measurements were within a 4 mDa agreement with the expected [C₂₇H₄₅]⁺ ion, m/z 369.3521.

Analytical Comparison of Animal-Derived and Synthetic Cholesterol (SyntheChol™)

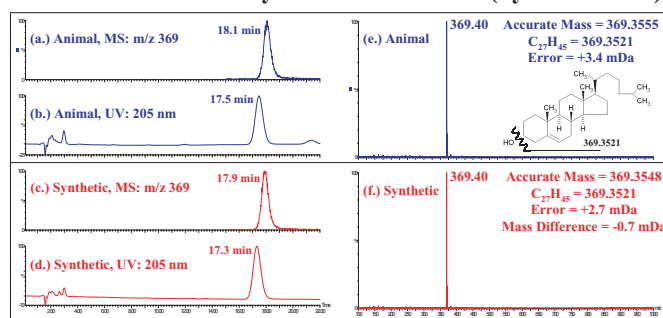


Figure 2. MS- and UV-detected HPLC chromatograms of animal-derived (a-b) and synthetic (c-d) cholesterol. The corresponding background subtracted mass spectra are also shown (e-f). Both spectra display a base ion at m/z 369.40. The proposed fragmentation producing this ion is shown. Accurate mass measurements were performed, and indicate that the difference between animal-derived and synthetic cholesterol was only 0.7 mDa. The chromatographic data and accurate mass measurements confirm the two analytes are identical species.

In Figure 3, the SyntheChol NS0 Supplement (Product Code S5442) is then shown to perform as well as SyntheChol dissolved in ethanol and is offered as a sterile, ready to add supplement with increased stability in cooler conditions. This has been demonstrated in spinner flask culture and bioreactors.

SyntheChol NS0 Supplement (Sigma S5442) Support NS0 Cell Growth in Spinner Flask and Bioreactor

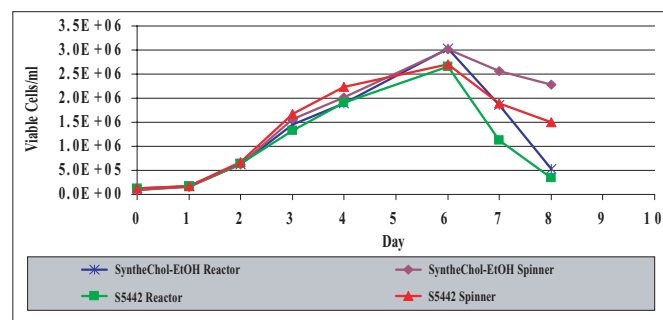


Figure 3. Comparison of NS0 cell growth in spinner flask and bioreactor with SyntheChol-EtOH and SyntheChol NS0 Supplement (Sigma S5442). NS0 cell growth was tested with Sigma hybridoma medium (H4409) supplemented with 5 mg/L of SyntheChol dissolved in ethanol or supplemented with SyntheChol NS0 Supplement (Sigma S5442). Both formats of SyntheChol support similar cell growth in spinner flask and 5 L bioreactor.

The SyntheChol NS0 Supplement is further shown to be as good as or better than a competitor's equivalent product with regard to growth of NS0 recombinant cells. When used with an IgG producing NS0 derived recombinant clone the SyntheChol NS0 Supplement is as good as or better than the competitors' product with regard to IgG production (as shown in Figure 4). This data is further confirmed by the data obtained from a testing of the second recombinant NS0 clone (as shown in Figure 5).

Cell Growth and rIgG Production of NS0 Recombinant Clone 1 with SyntheChol NS0 Supplement (Sigma S5442)

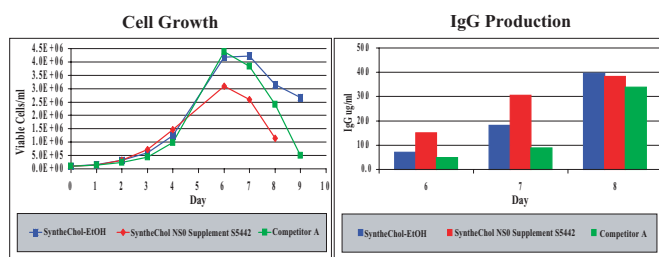


Figure 4. Cell growth and rIgG production of NS0 recombinant clone 1 were compared using the medium supplemented with SyntheChol-EtOH, Competitor A product and SyntheChol NS0 Supplement (Sigma S5442). NS0 cell growth was tested with Sigma hybridoma medium (H4409) supplemented with 5 mg/L of SyntheChol dissolved in ethanol or supplemented with SyntheChol NS0 Supplement (Sigma S5442). Although the cell growth is slightly lower in the medium with SyntheChol NS0 Supplement, the total IgG production and specific IgG productivity is higher or comparable to other tested samples in the NS0 recombinant clone 1.

Conclusions

- Sigma-Aldrich Corporation has successfully produced the first non-animal derived cholesterol called SyntheChol (Product Code C1231). This product promotes the growth of NS0 and NS0 derived cell lines in a medium totally devoid of animal derived components.
- SyntheChol NS0 Supplement (Product Code S5442) enhances the solubility of the SyntheChol molecule for easier use. This supplement is supplied as a 500X concentrate, is sterile filtered, and ready for use. It is specifically formulated for use in conjunction with the Hybridoma Medium, Animal Component Free (Product Code H4409).
- SyntheChol NS0 Supplement provides similar or better growth and rIgG production as compared with SyntheChol dissolved in ethanol and a leading competitor's product.

Cell Growth and rIgG Production of NS0 Recombinant Clone 2 in SyntheChol NS0 Supplement (Sigma S5442)

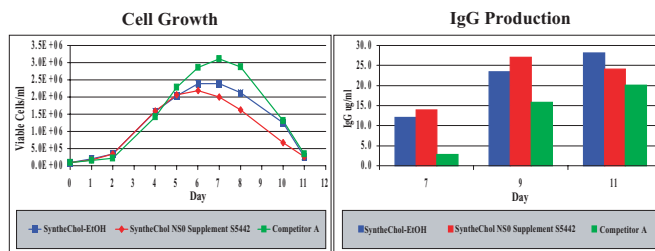


Figure 5. Cell growth and rIgG production of NS0 recombinant clone 2 were compared using the medium supplemented with SyntheChol-EtOH, Competitor A product and SyntheChol NS0 Supplement (Sigma S5442). NS0 cell growth was tested with Sigma hybridoma medium (H4409) supplemented with 5 mg/L of SyntheChol dissolved in ethanol or supplemented with SyntheChol NS0 Supplement (Sigma S5442). Although the cell growth is lower or slightly lower in the medium with SyntheChol NS0 Supplement, the total IgG production and specific IgG productivity is much higher than that in other tested samples in the NS0 recombinant clone 2.

