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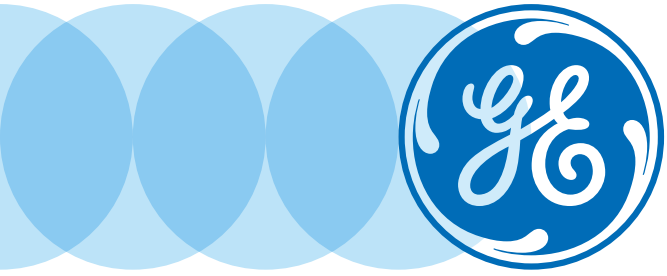

# Lipids in cell culture media

**Lipids function in cell systems as energy stores, metabolic intermediates, components of cellular membranes, and in transport/signal systems. Biomanufacturers often supplement cell culture media with identified lipids for better performance and efficiency, in a desire to avoid serum, and to support special requirements of particular cells or manufacturing processes. Lipids that culturists use include fatty acids, sterols, triacylglycerols, and glycerophospholipids. Medium supplementation with lipids presents a unique issue: their limited solubility makes adding sufficient quantities a challenge. Of the many potential ways to disperse lipids, the successful approaches employed in medium supplementation are complexation of the lipid with a soluble carrier, devising a self-assembly to the required particle size, and producing an emulsion particle of sufficient size and stability.**

## Introduction

Requirements for better performance, a desire to avoid serum, and improved understanding of culture systems are inspiring fresh interest in supplementing cell culture media with identified lipids. Surprisingly, there is no universally accepted definition of lipids. Originally, scientists considered all naturally occurring compounds that are soluble in non-polar solvents such as benzene, to be lipids. For cell culturists, a more practical definition is water-insoluble biomolecules, biosynthetically or functionally related to fatty acids and their derivatives. This definition includes fatty acids, sterols, triacylglycerols, glycerophospholipids, and sphingolipids, but excludes steroid hormones, fat-soluble vitamins, and petroleum products. There is logic to both definitions, and either will suffice in this article. By any definition, cell culturists have a special concern about lipids: their limited solubility in cell culture media makes adding sufficient quantities a challenge.

Lipids function in three roles: they serve as energy stores, as structural components of cellular membranes, and in transport and signal systems. Cell and organelle membranes contain much of a cell's lipids. Major categories of biological lipids include glycerophospholipids (e.g., phosphatidyl choline), sterols (e.g., cholesterol in animals and phytosterols in plants), sphingolipids (e.g., ceramides), and various lipoprotein complexes. Structural functions of many lipids, such as the requirement of cholesterol for animal cell membrane fluidity, have been extensively described. Many signaling systems rely upon lipid-containing complexes, such as the familiar AB0 blood type determinant from the lacto- and neolacto-series of glycosphingolipids.

## Cellular requirements

In any living system an essential nutrient is a compound that the organism requires for growth and reproduction, and which the organism cannot produce. Organisms can synthesize most of the dozens of lipids they require for primary cellular functions. In mammals, though, two fatty acids (linoleic and α-linolenic acid) have been proven to be essential.

For decades scientists have reported on the lipid requirements of particular cell lines as cultured in an excess of lipids, usually from fetal bovine serum (FBS). With the more widespread use of serum-free medium (SFM) and chemically defined medium (CDM), researchers are now looking at the requirements of cells maintained in minimal complements of identified lipids. Also, a practical distinction is emerging between those components that are required for culture maintenance and those components, and their levels, which promote optimal performance. Apparently, much of the work performed with cells adapted to media with high lipid levels provided an inaccurate picture of the actual requirement of cells in culture. Many known SFM formulations lack linoleic or α-linolenic acid, yet sustain indefinite cell growth and full function. It appears that these essential fatty acids are, in fact, not essential for most animal cell culture (1).

Supplementation of cell culture systems with particular non-essential fatty acids, phospholipids, and sterols significantly improves performance. Providing cells with appropriate preformed lipids, even when not essential, reduces the need for their biosynthesis by the cell. The result is more efficient metabolism, which is especially evident where the rate of cell division is important or where the cell produces high

**Table 1.** Features of lipid supplementation approaches

| | Physical stability | ADCF* potential | PF† potential | CD‡ potential | Active lipid capacity | Formulation adjustable |
|---|---|---|---|---|---|---|
| Serum | High | No | No | No | High | Minimally |
| Serum extracts | High | No | No | No | High | No |
| Solvents | High | Yes | Yes | Yes | Low | Yes |
| Albumins | High | rAlbumin | No | Nearly | Medium | Somewhat |
| Emulsions | Low | Yes | Yes | Yes | High | Significant |
| Micelles | High | Yes | Yes | Yes | Medium | Somewhat |
| Liposomes | Med | Yes | Yes | Yes | Medium | Somewhat |
| Cyclodextrin | High | Yes | Yes | Yes | High | Yes |

* Animal-derived component-free

† Protein-free

‡ Chemically defined

levels of transgenic product (2). Some cultured cells are truly auxotrophs for particular lipids, meaning that these lipids are essential to them. For example NS0, a common myeloma cell line, requires large amounts of cholesterol, but is incapable of producing it. This unusual phenotype is caused by the silence of an enzyme in the cholesterol synthesis pathway.

## Medium supplementation

People have been devising heuristic formulas for working with lipid dispersions for millennia. Churning butter and adding an egg to a failing recipe are examples of applying simple techniques without necessarily understanding the chemistry behind them. At the other extreme, modern industries such as the pharmaceutical, food processing, agrochemical, and cosmetic industries often approach oil and water dispersion issues with sophisticated technologies. Interfacial and colloid chemistry, hydrocarbon chain packing, and lyotropic and thermotropic mesophase behavior have all been brought to bear against lipid dispersion issues (3).

Whatever approach or combination is used, the goal of cell culture medium supplementation is to disperse select lipids such that they are non-toxic, are taken up by the cells in a controlled fashion, can be micro-filtered, and remain stable upon storage at 5°C for up to a year.

These requirements can be met in three ways: 1) adsorb the lipid to a soluble carrier molecule, 2) devise a formula that drives lipid self-assembly to the required particle size, and 3) disperse and stabilize a lipid mixture to a particle of sufficient transient size and stability. Each of these approaches is a science in its own right, and all have been used in developing cell culture media (Table 1).

## Adsorption to a carrier

Animal serum, the original supplement which provided lipids to cells in culture, uses proteins as carriers of every lipid required by mammalian systems. FBS, the most common serum in cell culture, contains high levels of lipids. For example, FBS contains approximately 300 µg/mL cholesterol and 30 µg/mL oleic acid. The major lipid carrier protein in serum include albumin, a globular protein with many distinct hydrophobic moieties, represents over 50% of the protein in serum, and is a very efficient vehicle of fatty acids and sterols; and four classes of apoprotein-containing lipoproteins: chylomicrons that carry triacylglycerides, very low-density lipoproteins (VLDL) that carry fatty acids, and low- and high-density lipoproteins (LDL and HDL, respectively) that are principally involved in cholesterol transport.

The high level and diversity of lipids in serum is sometimes detrimental. For example, cells cultured in serum are constantly exposed to a number of steroids, making it difficult to determine the specific effects of any particular one.

Serum extracts and lipid-rich fractions are popular products for adding high concentrations of serum lipids to media. Commercially available fractions of animal serum contain a number of serum lipids, including cholesterol, fatty acids, and phospholipids bound to select serum proteins. While the ratio of lipids remains fixed, these IgG-free and albumin-reduced supplements allow variation of the total lipid concentration and combination with other supplements. IgG-free and albumin-reduced supplements are of significant value in some serum-dependent systems by, for example, reducing material costs and improving performance. Notably, they

have been shown to provide no benefit when added to many existing SFM formulations. However, recent reports point to significant value if this supplementation is combined with albumin. This is a good example of a practical/formulistic solution to a lipid dispersion issue. As the serum extracts are quite stable on their own, it appears that the benefit albumin is specifically providing here is its capacity for lipid transport (4), or in this case, lipid particle dispersion-to-cell delivery.

Bovine serum albumin (BSA) is a common vehicle for lipids in medium formulations. BSA is commercially available in a variety of purities and formats. All basic preparations contain high levels of serum lipids, especially fatty acids and phosphatidylcholine. It is possible to create or purchase a reduced-lipid BSA. Such a preparation has the capacity to adsorb a significant amount of added lipids of choice.

Polar organic solvents such as ethanol and dimethylsulfoxide that demonstrate the lowest inherent toxicity are commonly used as carriers of lipids. These solvents work well as vehicles of low levels of most lipids, producing sterile filterable and stable dispersions. However, they suffer from the two major limitations of significant inherent cellular activity: toxicity and limited capacity.

Cyclodextrins, naturally occurring circular polymers of glucopyranose, increase the solubility of lipids (Fig 1). The function of cyclodextrins is similar to other adsorptive systems, in that they encase or chelate lipids in a more water-soluble molecule, and thereby increase the lipids' aqueous solubility. The addition of various side groups to the cyclodextrin molecule increases its solubility in water to nearly 50% (w/v), while leaving the hydrophobic, lipid-active central cavity intact.

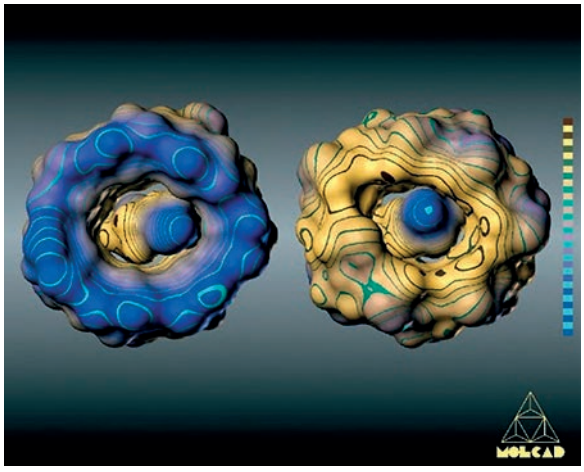


**Fig 1.** Molecular model of β-cyclodextrin inclusion complex. Guest molecule is 1,4-butanediol, a vitamin B6 precursor with both hydrophilic (blue) and hydrophobic (yellow) regions. Reprinted with permission of Stefan Immel, Technical University Darmstadt, http:caramel.oc.chemie.tu-darmstadt.de/immel/.

Two products, HyClone™ LS1000 and HyClone LS250, successfully utilize cyclodextrin to solubilize cholesterol and fatty acids for supplementation of cell culture media. LS1000 is designed to be added at culture initiation, or as a component in fed batch cultures (Fig 2). LS250 is optimized for medium supplementation prior to or at culture initiation. Dilutions in media can be filtered and are stable in appropriate storage for months.

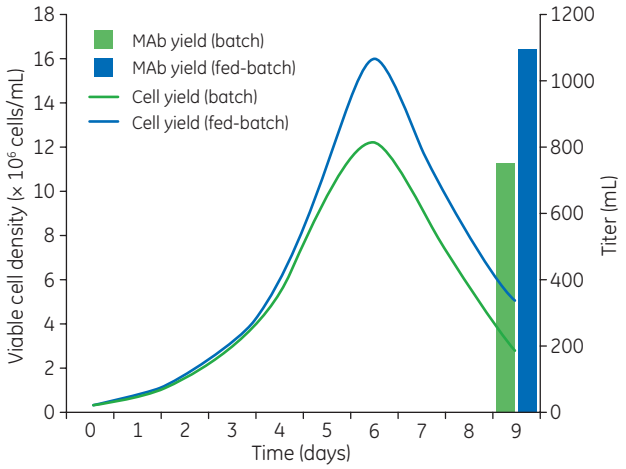


**Fig 2.** rGS NS0 cells cultured in CDM4NS0 with and without day 3 feeding with LS 1000 cyclodextrin-based lipids and standard GS supplement.

## Phase-based dispersions

Liposomes, emulsions, and microemulsions are forms of lipid dispersions used in medium supplementation.

### *Liposomes*

Spheres of lipids in the lamellar phase with an aqueous core are variously referred to as lipid bilayer vesicles or liposomes. All nutrient lipids carried by liposomes must be components of the lipid lamellae. It is sometimes possible to combine lamellae-forming polar lipids with nutrient lipids such that the phase behavior and intrinsic curvature of the mixture generate liposomes of suitable size and stability. For example, cholesterol or fatty acids can intercalate within the acyl chains of polar lipids, such as phosphatidylcholine. The temperature, pH, and tonicity of cell culture media are usually maintained within a narrow range. This allows the use of technologies based on such principles as steric hindrance, hydrogen bonding, and electrostatic charge to be exploited in developing stable preparations. Although suitable mixtures of polar and nutrient lipids so formulated are conceivable, published attempts mostly report failure. It should be mentioned here that there are newer technologies available that employ structures similar to liposomes but composed of lipids in lyotropic phases other than the lamellar, such as the hexagonal and cubic phases.

### *Emulsions and microemulsions*

These two related forms of filterable dispersions can have a term of physical stability comparable to that of the medium itself.

Emulsions are kinetically (non-equilibrium) stable dispersions. They are produced by first reducing the particle size through introduction of hydrodynamic force, and then stabilization of the particle surface. Addition of such amphophiles as polar lipids and certain peptides or polymers, and modulations of the emulsion particle surface charge are common ways of introducing a stabilizing structure to their surface.

Microemulsions can be formed by using detergents to generate mixed micelles, containing the lipid of interest at their core. The key to developing such microscale and thermodynamically stable dispersions is to find a surfactant that is neither too toxic nor too disruptive to cell membranes. The surfactant and lipids must form filterable structures of acceptable size and stability at normal temperatures. Concepts such as the critical micelle concentration (CMC) and hydrophilic/lipophilic balance (HLB) are useful here.

A number of SFM formualtions in current use have their lipid components dispersed by these means. In both cases, amphiphiles are often used to surround and stabilize a particle of lipid, presenting their hydrophobic region to the lipid and their hydrophilic region to the aqueous medium.

## Materials

Nutrient lipids are available from a variety of sources and vendors. Enriched lipid fractions extracted from such diverse starting materials as animal serum, sheep wool, fish oil, and soybeans are commercially available. Individual lipids purified from these naturally occurring rich sources are also available. Chemical synthesis and derivatization is used to produce those lipids that are either rarely found in nature or that are abundant only in unacceptable sources. For example, in formulating animal-derived component-free (ADCF) media, cholesterol, commonly obtained from sheep wool, must instead be synthesized from non-animal origins.

Tween™ 80 (a non-ionic detergent), Pluronic™ acid (a block co-polymer), phosphatidyl choline (or lecithin), and protein hydrolysates, such as those from beef or soy, have proven to be the most popular materials for dispersing non-polar lipids in serum- and protein-free cell culture media. Lipid particle surface and interfacial energies must be overcome in both the generation of meta-stable emulsions and in accelerating the equilibrium of microemulsions. This is accomplished in instruments capable of generating extreme hydrodynamic force while minimizing heat production (e.g., the EmulsiFlex™-C50, Avestin, Ottawa, ON). The addition of chemical antioxidants, such as α-lipoic acid and α-tocopherol, can reduce the peroxidation of polyunsaturates in the formulation. Procedures that limit the introduction of free oxygen also help in this regard. Interestingly, many lipids seem to be exquisitely protected from oxidation when complexed with cyclodextrin (5).

## Applications

Although there are tens of thousands of distinct lipid compounds in natural systems, relatively few are used in serum-free medium formulations. Most of the more successful formulations are proprietary, so exact recipes cannot be obtained. Also, there has been an evolution of thought about which lipids are beneficial. For example, supplementation with steroids (such as testosterone), and polyunsaturates (such as arachidonic acid), which followed interest of their animal system functions, has diminished of late. Lipids currently referred to in the literature for supplementation include cholesterol, cod liver oil, soybean oil, as well as oleic, linoleic, and palmitic acids. Applications for lipid-based medium supplementation include 1) clone-specific essential and performance-enhancing lipids in most CD formulations, 2) special requirements, such as the need for high levels of cholesterol in NS0-based transgenic producers, and 3) fed-batch procedures in bioreactors (6).

## Conclusion

Systems for delivering lipids have evolved as the culture requirements of research and industry changed. Earlier, supplementation with animal serum provided an effective answer. As concern about animal-derived products increases, medium producers move further towards SFM, ADCF, and CDM technologies. The elimination of serum, with its complement of natural lipid carriers, initially posed significant problems to medium supplementation. Fortunately, these are being solved through the approaches and techniques outlined here.

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