Testing Times

By using the design of experiments approach in the production of chemically-defined and non-animal-derived media, simultaneous testing of various compounds can help to determine potential interactions, as well as individual effects

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Mammalian cells are one of the most widely-used platforms in the production of biopharmaceuticals. Among them, Chinese hamster ovary (CHO) and human embryonic kidney (HEK 293) cells are extensively used for the production of recombinant proteins, vaccines and viral vectors.

In the manufacturing of biopharmaceuticals, the use of serum-free medium has grown significantly in industrial applications where the use of bovine serum represents a safety hazard, as well as a source of unwanted contamination. Furthermore, the use of non-animal-derived and chemically-defined culture media has become more generally adopted. This is because culture media used for animal cell cultures is very complex. Historically, serum has been a crucial component of their composition as a provider of multifaceted biological molecules, such as hormones and growth factors, as well as numerous low molecular weight nutrients (1).

The emergence of industrial-scale mammalian cell culture for the production of protein pharmaceuticals has presented a new challenge for cell culture medium design, and quality control aspects have arisen from the use of foetal bovine serum (FBS). The issues of reliability of supply, variability in performance and the risk of biological contaminants (mycoplasmas and viruses) has created serious safety concerns among regulatory agencies. These are further increased by the emergence of prion-related diseases – specifically bovine spongiform encephalomyelitis. This has led to a rising use in chemically-defined non-animal-sourced medium components to replace both serum and medium supplements purified from animal sources, such as insulin, transferrin and albumin (2-4).

Protein Types

Insulin serves as a growth and maintenance factor, and is considered to be important for serum-free cultures (5). It stimulates uridine, glucose uptake and synthesis of RNA, proteins and lipids, and also increases fatty acid and glycogen synthesis (6).

Transferrin is one of the most essential growth-promoting supplements in serum-free medium, and its omission causes severe inhibition of cell growth (7). It is an iron-binding glycoprotein that interacts with surface receptors and is closely related to the transport of iron across the plasma membrane (8). Transferrin also has additional *in vitro* functions – for example, chelation of deleterious trace materials that are unlikely to be replaced by other components.

Selenium is a trace element essential for mammalian cell cultures (9). Its mechanism is poorly understood, although there is evidence that selenium enhances growth rate in serum-free cultures (10). Lipids are required for proliferation, differentiation and antibody secretion. They play a major role in the cell membrane, which is composed of a phospholipid bilayer, and help in the transmission of

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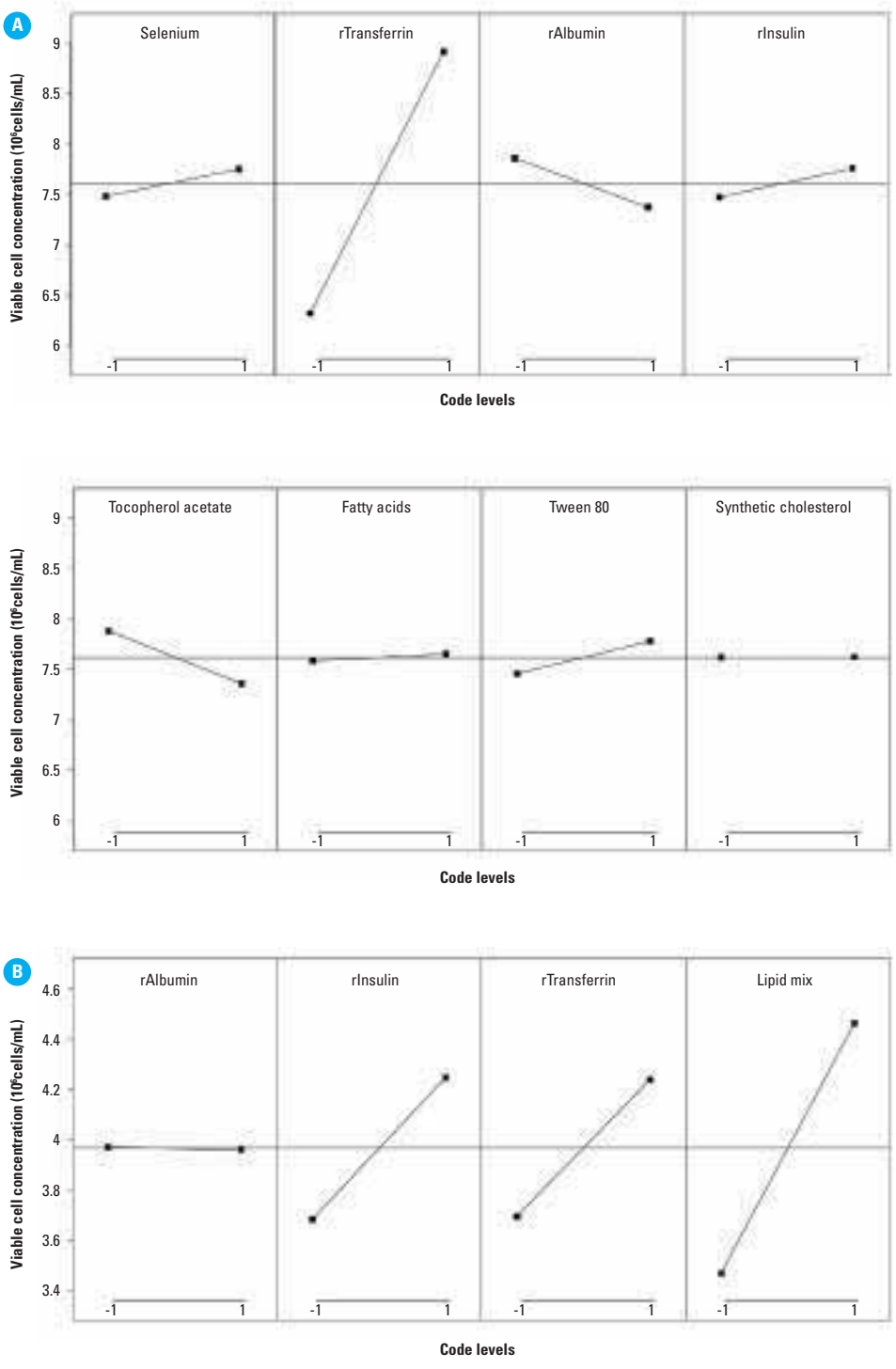


Figure 1: Results for the screening step in the selection of supplements using the Plackett-Burman methodology. Positive slopes indicate a positive effect of a particular supplement.

Part A results obtained for the CHO cell line with FreeStyleCHO medium.

Part B results obtained for HEK 293 cells with FreeStyle medium

nutrients into the cell and excretion of proteins out of the cell (11).

Albumin – most commonly known as human serum albumin – prevents the toxic effect of free fatty acids on cells in culture, acts as a metal ion binding protein, and has antioxidant effects (12).

Design of Experiments

The optimisation of the media composition – in terms of selecting the most appropriate components, as well as their ideal concentration – can be performed using design of experiments (DoE).

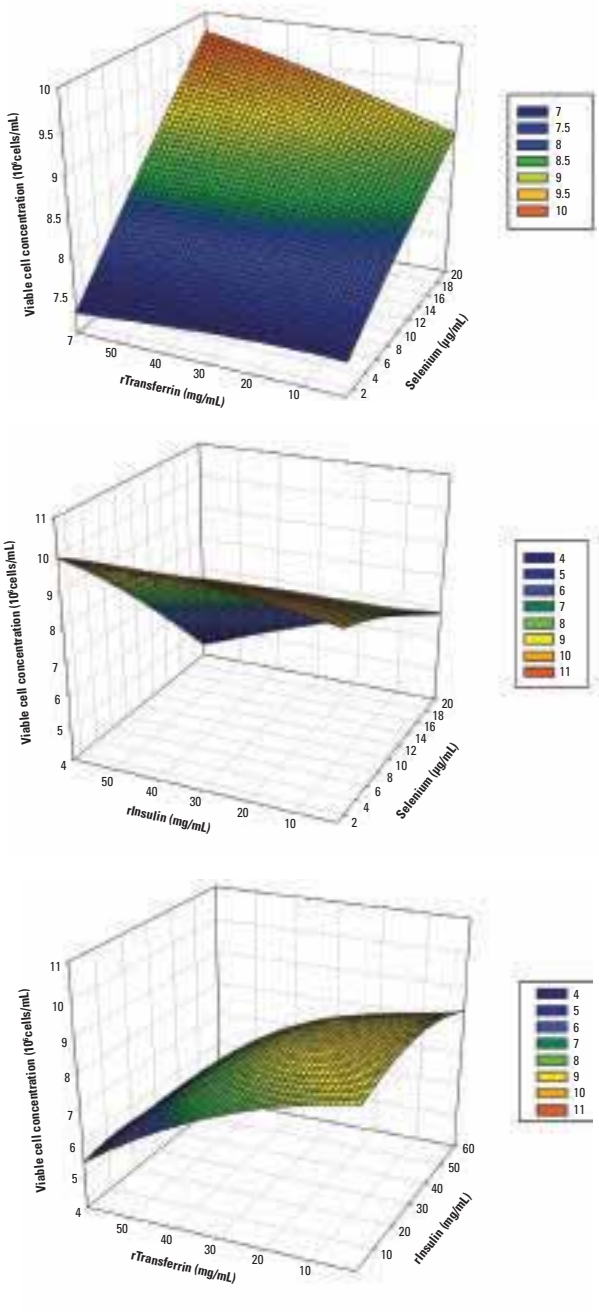
DoE is a useful method that allows for the simultaneous determination of the individual and interactive effects of many factors that could affect the response function (13).

It can be divided into two steps: one for screening the most effective compounds; and one to optimise their concentration – for example, to support cell growth.

The Plackett-Burman design is a two-level multifactorial design based on the rationale known as balanced incomplete blocks. Using this, up to N-1 components can be studied in N experiments, where N must be a multiple of four (14). The two levels tested are usually defined by performing a series of toxicity studies for each individual compound. The dose-response effect of a given compound on cell viability is then studied, and the upper limit for the screening tests is set at the concentration showing a clear decline in the viability curve.

After finding the most relevant factors that influence cell growth, the next step is to enhance the concentrations of these components in the growth medium. Response surface methodology (RSM) is a powerful experimental tool for seeking the optimum conditions for a multivariable system, and is a favourable technique for optimisation (15). It comprises mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables, and also generates a mathematical model describing the overall process (16).

Figure 2a: Results for the optimisation step using the Plackett-Burman methodology. Surface responses show the combined effect of components. Results obtained for the CHO cell line with FreeStyleCHO medium



The Box-Behnken design is a type of RSM which combines 2k factorials with incomplete block designs. The resulting designs are usually very efficient, as they require few experiments and allow for the determination of combined effects of different factors.

Case Study

In an experiment, CHO S cell growth optimisation was carried out using the chemically-defined medium FreeStyleCHO.

Up to eight non-animal-derived compounds were tested as supplements – recombinant insulin (rInsulin), recombinant transferrin (rTransferrin), recombinant albumin (rAlbumin), selenium, tocopherol acetate, synthetic cholesterol, tween 80 and fatty acids – in order to increase maximum cell density. It is generally recognised that the expression of a recombinant protein in CHO cell cultures is directly proportional to the integer of viable cells of the growth curve.

Plackett-Burman initial screening experiments indicated that only three of the supplements (rInsulin, rTransferrin and selenium) had positive effects on cell growth – as seen in Figure 1, part A (page 36). However, in spite of tween 80 having a positive slope, and therefore an individual positive effect, it was discarded for the next round of optimisation due to its negative interaction with some of the other supplements tested.

The concentration of the three selected components was further optimised by means of a Box-Behnken design, and surface responses are presented in Figure 2a. A maximum cell density of 10 x 10^6 cells/mL in batch mode was achieved for FreeStyleCHO medium supplemented with selenium (1µg/L), rTransferrin (20.2 mg/L) and rInsulin (26.1 mg/L), while only 8 x 10^6 cells/mL could be achieved with unsupplemented medium.

For HEK 293 cells, two aspects were tested: cell growth as well as efficiency of transient transfection. In general, transient transfection is used at the process development level when relatively small quantities of a given molecule need to be produced rapidly for structural and functional testing, including preclinical trials. Transient transfection in this particular case was performed to produce HIV-1 Gag-GFP virus-like particles (VLPs). The medium selected was FreeStyle, and supplementation with non-animal-derived components included rInsulin, rTransferrin, rAlbumin and a mixture of lipids. Plackett-Burman initial screening experiments indicated that only three of the supplements (rInsulin, rTransferrin and lipid mix) had positive effects, as seen in Figure 1, part B (page 36).

The concentration of these components was further optimised by means of a Box-Behnken design, and surface responses are presented in Figure 2b. Such optimisation resulted in improved HEK 293 cell growth and VLP production for optimal concentrations of rInsulin (19.8mg/L), r-Transferrin (1.6mg/L) and lipid mix (0.9X).

The maximum cell density attained using the optimised culture medium was 5.4 × 10^6 cells/mL in batch mode – almost double that observed using the unsupplemented medium (2.9 × 10^6 cells/mL) (see Figure 2b). Best production performance was attained when cells were transfected at mid-log phase (2-3 × 10^6 cells/mL) with medium exchange at the time of transfection using standard amounts of plasmid DNA and polyethylenimine (17). By using optimised production protocol, VLP titers were increased by 2.4, therefore obtaining 2.8µg of Gag-GFP/mL corresponding to approximately 8.2 × 10^9 VLPs/mL.

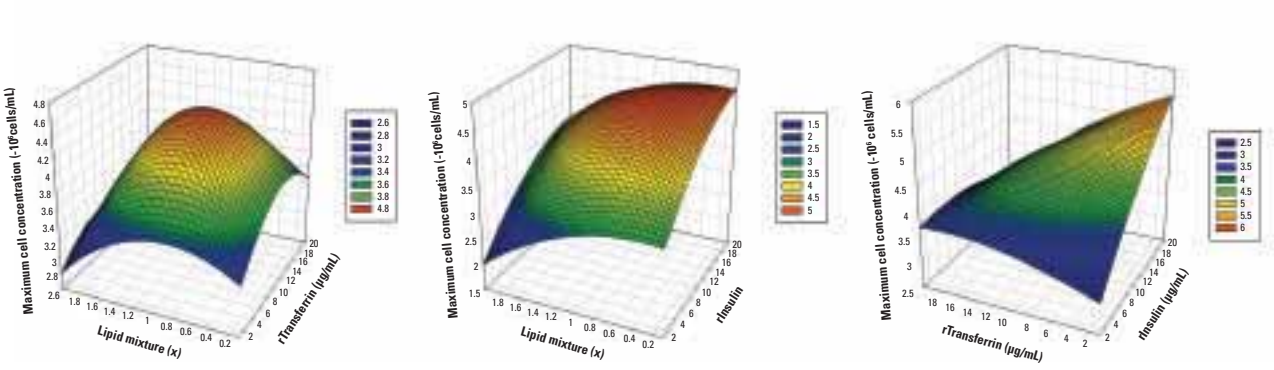


Figure 2b: Results for the optimisation step using the Plackett-Burman methodology. Surface responses show the combined effect of components

Results obtained for HEK 293 cells with FreeStyle medium

Optimal Conditions

In conclusion, two study cases have outlined an approach to optimise a given chemically-defined commercial cell culture medium by its supplementation with non-animal-derived compounds. The use of DoE as a tool to accelerate both the screening of the most efficient compounds and the determination of the corresponding optimal conditions is very effective. For CHO S cells, the optimal conditions were found for FreeStyleCHO medium supplemented with selenium (1µg/L), rTransferrin (20.2mg/L) and rInsulin (26.1mg/L); while for HEK 293 cells, the optimal conditions were found for FreeStyle medium supplemented with rInsulin (19.8mg/L), rTransferrin (1.6mg/L) and lipid mix (0.9X).

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About the author

Francesc Gòdia is a Professor of Chemical Engineering at Universitat Autònoma de Barcelona (UAB) where his research activity is focused on biotechnology and biochemical engineering, specifically the culture of mammalian cells in the production of proteins. Previously, he served as Head of the Chemical Engineering Unit at UAB and coordinated the Chemical Engineering degree, and was President of the Spanish Society for Biotechnology. Francesc is author of more than 80 papers, holds 50 patents, and is one of the founders of HEXASCREEN Culture Technologies, which focuses on the development of single-use minibioreactors for screening in biotechnology.

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