Batch effect adjustment to lower the drug attrition rate of MCF-7 breast cancer cells exposed to silica nanomaterial-derived scaffolds

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Abstract

Drug attrition rate is the calculation or measure of the clinical efficacy of a candidate drug on a screen platform over a specific period. Determining the attrition rate of a prospective cancer drug is a reliable way of testing the clinical efficacy. A low attrition rate in the last phase of a preclinical trial increases the success of a drug discovery process. It has been reported that the attrition rates of antineoplastic drugs are much higher than for other therapeutic drugs. Among the factors identified for the high attrition rates in antineoplastic drugs are screen based platforms involving human-derived xenografts, extracellular matrix (ECM) derived scaffold systems, and synthetic scaffolds, which all have propensity to proliferate tumour cells at faster rates than in vivo primary tumours. Other factors that affects the high attrition rates are induced scaffold toxicity and assays that are irrelevant but affects data processing. These factors contribute to the wide variation in data and systematic errors. As a result, it becomes imperative to filter batch variations and to standardise the data. Importantly, understanding the interplay between the biological milieu and scaffold connections is crucial as well. Here the cell viability of MCF-7 cells exposed to different scaffolds were screened prior to cisplatin dosing using the calculated p-values. The statistical significance (p-value) of data was calculated using the one-way ANOVA, with the p-value set as : $0 < p < 0.06$. Additionally, the half-maximal inhibitory concentration (IC₅₀) of the different scaffolds exposed to MCF-7 cells were calculated with the probit extension model

and cumulative distribution (%) of the extension data. The chemotherapeutic dose (cisplatin, 56 mg/m²) reduced the cell viability of MCF-7 cells to 5% within 24 hours on the scaffold developed from silica nanoparticles (SNP) and polyethylene glycol (PEG) formulation (SNP:PEG) mixtures with a ratio of 1: 10 respectively.

Keywords: Drug attrition rate, Temperature induced batch effects, Batch effects, Data analytics, Probit analysis, Nanomaterial scaffolds, Time-dependent cellular responses

1. Introduction

For several decades, researchers have used *in vitro* technologies as models for drug discovery. However, some of these approaches raise concerns due to high attrition rates in the clinical development phase.^{1, 2} It is well established that scaffolds capable of differentiating cells into three-dimensional structures are more promising as drug discovery platforms.^{1, 3 4, 5, 6-8}

This work investigates the process of removing batch effects from breast cancer cells of MCF-7 model systems exposed to scaffolds derived from silica nanoparticles. Batch effects,⁹ are the systematic, non-biological differences in sample preparations in microarray experiments that contributes to data, and makes the data incomprehensible for cross-batch prediction performance. The scaffolds were developed at room temperature using mixing and slow evaporation.¹⁰ The data was processed by removing the batch effect¹¹ due to temperature variation over time on the scaffolds. In this study, the batch effect is a non-biological factor that contributes to the biological response. After processing the cell viability data at 24 hrs and 48 hrs, the probit analysis was used to calculate the significance of the data.

Probit analysis is a statistical tool for analysing bioassay experiments, applicable mostly to data sets with a dependent, single response variable. Often, there may be variables within a data set that do not correlate with the standard probit analysis but require an extension model.¹² Probit extension models have been used to solve clustered data graphs, such as to discover the underlying structure within a data set,¹³ synchronise multiple data sets within a mixed data set using mixed-effects models,¹⁴⁻¹⁶ assess the level of toxicity in bioassay experiments,^{17, 18} etc.

Here, a simple algorithm for solving bioassay selection involving scaffolds have been proposed. This study circumvents the complex mathematical processes in probit extension models. The algorithm model includes batch adjustment, standardisation, and statistical considerations for extending a probit model of a composite scaffold.

2. Method

The following reagents were purchased from Sigma-Aldrich, Australia: Ludox AS-40 colloidal silica nanoparticles (SNP), polyethylene glycol, (PEG-8000) and polyamidoamine first generation (1^o PAMAM) dendrimer. The following culture media reagents were purchased from Thermofisher Scientific, Australia: Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), TrypLE™ Express Enzyme (1X) with no phenol red and Hanks balanced salt solution (HBSS). The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega, Australia. The tissue culture flasks (25 cm², Corning) were purchased from Invitrogen, Australia. The green, fluorescent cytopainter (ab176735) was purchased from Abcam, Australia. The Neubauer hemocytometer blood counting chamber was used for all the cell counting. Cisplatin was purchased from Tocris Bioscience (Minneapolis, USA) and supplied by Invitrogen, Australia.

The bioassay experiments consist of composite substrates derived from the following: silica nanoparticles (SNP), PEGylated SNP (SNP: PEG), dendrimer mixed with PEGylated SNP (SNP: PEG: Dendrimer), *Terminalia ferdinandiana* Excell (Kakadu plum) leaf methanol extract (KPLM), mixed with PEGylated SNP (SNP: PEG: KPLM) and *Terminalia ferdinandiana* Excell (Kakadu plum) ethyl acetate fruit extract (KPFE), mixed with PEGylated SNP (SNP: PEG: KPFE). The American Type Culture Collection (ATCC) MCF-7 cell line was the breast cancer model selected for this study. All substrates were made sterile at the time of cell culture experiment by exposing to UV radiation source in the cell culture laminar flow cabinet for 45 minutes before seeding the cells. A volume of 100 µL (10³ cells/mL) was added on the substrates and incubated at 24 or 48 hrs in 96-well micro-test plates. All tests were performed in duplicate rows and repeated in duplicate, including the control on each plate. The cell viability of MCF-7 cells was evaluated using a proliferation assay kit (MTS reagent), purchased from Promega, Australia. A volume of 20 µL of MTS solution was transferred to each well and incubated for 1 hour before measuring absorbance at 490 nm using a Molecular Devices Spectra Max M3 plate reader. The MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) releases phenazine methosulfate (PMS) in the presence of viable cells, producing a formazan product with the absorbance maximum at 490 nm in phosphate-buffered saline.^{19, 20}

2.1 Preparation of nanocomposite substrates derived from silica nanoparticles

The stock solution (40 wt. %) of SNP was diluted with sterile deionised water across the range 40.0-1.0 (wt. %). The stoichiometric ratios of the two-composite mixtures (SNP and PEG) were formulated by adding the SNP concentrations to reciprocal PEG weight concentrations. The stoichiometric ratios

of the three-composite mixtures were obtained by mixing the two-composite mixture with a branched 1° PANAM dendrimer. The component mixtures were subsequently stirred at room temperature for 4 hours for even dispersion of both particles (SNP and PEG). The three-composite mixture was stirred for 24 hours at 4°C. A 100 µL volume of each mixture of the composite combinations was transferred individually to 96 well plates under low and high humidity conditions, according to the following specifications: Δ= 37°C, 15% humidity and 30°C, 90% humidity. The plates were wrapped with aluminum foil and stored at 4°C. Refrigeration of the substrates retained the surface conditions (results not shown).

2.2 Plant extraction

Terminalia ferdinandiana Exell, fruit and leaves were harvested and provided by NT Wild Harvest, Australia and were dried to achieve a constant mass following prolonged drying time. The extraction of the *T. ferdinandiana* fruit and leaf material followed previously reported protocols.²¹ Briefly, the Kakadu plum leaves and fruits were dried separately in Sunbeam food dehydrator and ground into fine powders. Individual 1 g masses of the powdered fruit and leaves were weighed into separate tubes, and extracts separated later, using methanol, deionized water, ethyl acetate, hexane, or chloroform to cover a wide fraction of bioactive compounds. The tubes were placed on a shaker for 24 hrs at 4°C. Each extract was subsequently filtered using Whatman number 54 filter paper under vacuum to remove particulate matter and the extracts were dried at 50 °C under vacuum. The resultant solid residues were dissolved in 10 mL of deionized water containing 1 % dimethyl sulfoxide (DMSO). The extracts were re-filtered through a 0.22 µm syringe filter (ThermoFischer, Australia) to remove particulates and sterilise the extracts before they were stored at 4°C until use.

2.3 Preparation of scaffolds derived from *T. ferdinandiana* extracts

Stoichiometric ratios of the two-composite mixtures (SNP and PEG) were diluted across the range 40.0-1.0 (wt. %) and the fruit and leaf extracts were added separately and mixed for 24 hrs at 4°C. A volume of 100 µL of the composite mixtures were then added to 96 well plates under low and high humidity conditions, according to the following specifications: Δ= 37°C, 15 % humidity and 30°C, 90 % humidity.

2.4 Addition of cisplatin to MCF-7 cells

A volume of 100 μL of cell culture containing 10^3 cells/mL was added to individual wells of a 96-well control plate. When the cells attained 80 % confluency, 20 μL of cisplatin was added to each well at the following concentrations: 7.5, 15, 22.5, 30, 37.5 and 45 $\mu\text{g/mL}$, and incubated for 24 hrs at 37 °C using 5% CO_2 in the culture chamber. A volume of 20 μL of MTS solution was added to each well and the plates incubated for another 1 hour. The absorbance was measured at 490 nm on a plate reader. The same procedure was repeated with the following substrate casts: PEGylated SNP (SNP:PEG), PEGylated SNP mixed with KPLM (SNP:PEG:KPLM), and PEGylated SNP mixed with KPFE (SNP: PEG: KPFE). All tests were performed in duplicate rows and duplicate controls on each plate. Sterile conditions of the substrate casts were obtained by exposing the plates to UV radiation for at least 30 minutes before the cell culture experiment.

2.5 Adjusting batch effect and standardising data on a nanocomposite substrate

The batch effect in this study describes the microstructural changes on substrate cast over time because of exposure to temperature changes. In this study, the batch effect of the substrate cast was adjusted at two levels. The first level was to filter the non-biological response from the direct absorbance reading of substrate and blank. The second level involved standardising the cell viability data.

For batch effect adjustment, the assumption was :

$$\sum_{i=1}^n x_s(c, t) = \sum_{i=1}^n (\mu - \bar{s})_{c,t} \quad (1)$$

$$\sum_{i=1}^n y(t) = \sum_{i=1}^n (\bar{y} - \bar{\phi})_t \quad (2)$$

$\sum_{i=1}^n x(c, t)$, are the concentration-time (c,t) dependent cellular responses upon exposure to the composite substrate (s); n is the number of samples, and x_s , is the cellular response with the substrate cast; μ , is the average cellular response; $\bar{s}$, is the mean response from the substrate cast without cells; $y(t)$, is the cellular response without the substrate cast. The growth is dependent on time t and on microstructural features of the scaffold matrix; $\bar{y}$, is the mean cellular response without the substrate cast, and $\bar{\phi}$, is the mean data from the empty well plate (blank).

$$\text{The cell viability assessment of the bioassay, } \varphi = \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,t}}{\sum_{i=1}^n (\bar{y} - \bar{\phi})_t} \quad (3)$$

After standardisation correction, the cell viability is expressed by:

$$\varphi = (k^*) \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,t}}{\sum_{i=1}^n (\bar{y} - \bar{\phi})_t} \times 100 \quad (4)$$

k^* , is the outlier correction factor, which is 0.5.

% cell mortality is expressed by:

$$\theta = 100 \% - (k^*) \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,t}}{\sum_{i=1}^n (\bar{y} - \bar{\theta})_t} \times 100 \quad (5)$$

2.6 Data correlation of the cellular responses on a nanocomposite substrate

Also assumed, $y(t)$ represents the total number of actively dividing cells on a 96-well plate (without modifying the plate), with incubation times of 24 or 48 hrs. At a given time, the first cycle population of y cells $\rightarrow 2y$. The second cycle population of cells $2y$ would have doubled to $4y$ with an exponential growth response, etc.

The cell proliferation (growth rate) for the control experiment is given by:

$$y(t) = y_o \times e^{kt} \quad (6)$$

y_o , is the total number of cells at the start of incubation; k is the growth rate when (>0), and inhibition rate when (<0). Applying a natural logarithm ($\ln$) to equation 6,

$$k = \frac{1}{t} \ln \frac{y(t)}{y_o} \quad (7)$$

The substitution of equation 2 into equation 7 provides the following equation:

$$k = k_{control} = \frac{1}{t} \ln \frac{\sum_{i=1}^n (\bar{y} - \bar{\theta})_t}{y_o} \quad (8)$$

The cell proliferation in the control plate was proportional to incubation time, indicating a time-dependent cellular response (within 48 hrs of incubation). The composite substrate casts followed a different metabolic pathway as measured by the cell viability. The substrate casts indicated proliferation for the first 24 hrs, and a growth inhibition after 48 hours, except for a dendrimer-based gel result (Figure 1).

The cell proliferation (growth rate) for the bioassay experiment with a composite substrate cast is therefore :

$$x_s(t) = y_o \times e^{kt} \quad (9)$$

From equation 9, there was an increased growth rate during the first 24 hrs incubation period, $k_1 > 0$. Conversely, a reduced growth from 24 to 48 hrs, $k_2 < 0$.

Applying a natural logarithm ($\ln$) to equation 9, the following equations are obtained:

$$k_1 = \frac{1}{t} \ln \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,24hrs}}{y_o} \quad (10a)$$

$$k_2 = \frac{1}{t} \ln \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,48hrs}}{y_o} \quad (10b)$$

The doubling time factor for the combined responses on a nanomaterial scaffold was estimated as a ratio of k_2 to k_1 .

The doubling time factor or proliferation frequency is given by:

$$f(t) = \frac{k_2(t_2)}{k_1(t_1)}, \text{ where for control, } k_2 > k_1 \text{ but for cells exposed to composite scaffolds, } k_2 < k_1, \text{ except for Fig. 1c} \quad (11)$$

2.7 Probit extension model to cellular responses on a nanocomposite substrate cast

Here, the standard probit method could not be used to correlate the cell viability data at 24 and 48 hrs. Hence, the following steps were used to complete the data processing before the probit transformation.

From equation 11, the cumulative frequency, cf :

$$cf = \sum_{i=1}^n f, \text{ within the data distribution} \quad (12)$$

Then the inverse of the cumulative frequency (cf^{-1}) was calculated. To correlate the data, every number in the distribution was divided by the highest number within the data set. The cumulative percentage of cell mortality for the combined responses, is given by:

$$\% \text{ cell mortality, } \theta_{24hrs,48hrs} = (1 - cf^{-1}) \times 100 \quad (13)$$

Transformed data was obtained with the standard probit table. Finally, the one-way ANOVA in the excel package (2016) was used to evaluate statistically relevant data (p-value). The cumulative distribution of the data (%) was obtained from equation 13 and later used for the calculation of the inhibition concentration of the different scaffolds exposed to MCF-7 cells.

2.8 Statistical analyses

The probit transformation data for the cell viability at 24- and 48 hrs, along with the computational analysis from the Microsoft program Excel (2016) software package, were used to analyse statistically relevant data; gauged by $0 < p < 0.06$ or 94% confidence interval limit. Additional screening requirements implemented into this study was the concept of : if the data set with values of p_1 or $p_2 \leq 0.06$, $p_3 > 0.06$, the data set will still remain non-significant, and if p_1 or $p_2 > 0.06$ and $p_3 \leq 0.06$, then the combination data set becomes statistically significant. The confidence interval limit makes allowance for batch variations that are inevitable in cell-based scaffold assays. The batch effect from the data was adjusted by filtering the non-biological response from the direct absorbance reading of

substrate and blank and standardising the cell viability data, including extending the data set for correction of data anomalies. The cumulative distribution data and the correlation coefficient data were calculated in Microsoft program Excel (2016). The error bars on the chart (Figure 4) is given by the relative standard deviation of 5.0 % (n=4).

3. Results and discussion

According to recent reviews,^{22, 23} phenotypic screening and target-based methods are the two main drug discovery approaches. The former involves *in vitro* assays to stop cell proliferation or reduce cancer cell size.²² Target-based assays use clone technologies to inhibit cancer biomarkers present in the tumor microenvironment.²³ The substrate platforms utilised in this investigation exhibits assay robustness, support inclusion of synthetic and biology moieties, and are sourced from cheap, synthetic, or natural plant material extracts. This study investigated the method for scaling down assay choices before adding cisplatin to the different scaffold systems.

The first data set involved the cell viability of MCF-7 cells exposed to synthetic scaffolds at 24-and-48 hours (Figure 1; Tables 1 to 6). As indicated from the figure and tables (Figure 1; Tables 1 to 6), the growth rate depended on the matrix formation and fabrication conditions of the substrate casts. The three-composite matrix containing SNP, PEG and first generation PAMAM dendrimer components had the highest cell growth responses for scaffolds produced under low and high humidity conditions. It was observed that the temperature and humidity conditions used to fabricate the different scaffolds contributed to the arrangement of cells to an extent, and to the cell viability. It was also observed that cells grown on scaffolds produced under high humidity conditions had higher proliferation rates (Figures 1a, b) for single and two composite scaffold systems. In contrast, the cell viability of MCF-7 cells exposed to a three-composite scaffold had the highest proliferation rates (Figure 1c). Since the cell proliferation on these scaffolds depend on the substrate topology, cells may also be inhibited, indicated in most of the results after 48 hrs incubation of MCF-7 cells. A comparative work referenced in this paper²⁴ displayed cell dynamic conditions between 29 °C and 37 °C using a modified polyacrylamide scaffold to grow cells. Even in an *in vivo* tumor environment, it has been shown that immune cells are capable of inhibiting cancer development or promoting more aggressive tumours.

25, 26

Thus, cancer cell behaviour is complex, requires a robust quantitative and qualitative data, and a cross-disciplinary approach is essential to understanding the research field. The responses in Figure 1 showed the effective growth of MCF-7 cells exposed to the different scaffold systems. The

proliferation frequency was calculated using equations 10 and 11. Then, from the data set, a probit extension model. The cell viability at 24 and 48 hrs were transformed to probit values and assigned with probits P1 and P2 respectively, and the combined responses (cell viability data at 24 and 48 hrs) were calculated from the probit extension model, probit P3 (Tables 1 to 6). Whilst the data sets with dependent, single response variables generally have good linear fits with probit values versus (vs) Logarithmic concentrations, Log_{10}C , there were no linear fits with the probit plots using the standard probits (probit values vs Log_{10}C) for the scaffolds exposed to MCF-7 cells (Figure 2). Notably, Figure 2 showed the probit profiles obtained from the standard plots (P1 and P2), and from the extended plot (P3). The advantage with the P3 plot is that incomprehensible data, indicated in Figures 2(B,C,F) from 24 and 48 were profiled as a single plot, which also applied to other figure plots indicated in Figure 2. Additionally, the statistical significance (p -value) of the data (from Figure 2) was evaluated with the regression analysis software in Excel 2006 programme using the compiled data from Tables 1 to 6. Table 7 was then used to estimate the p -values for P1, P2 and P3. Data sets with $0 < p \leq 0.06$ were considered statistically significant. Notably, the p -value (0.06) as opposed to 0.05 was used to make allowance for batch variations inevitable in cell-based scaffold assays. This approach was used to narrow down bioassay selection. Hence, only the PEGylated SNP (SNP: PEG) scaffold system fulfilled the screening requirement (Table 7), with $0 < p_1, p_2 \text{ or } p_3 \leq 0.06$. The probit values indicated statistically significant data with the calculated p -value (p_3). Additional screening requirements implemented into this study was the concept of : if the data set with values of $p_1 \text{ or } p_2 \leq 0.06, p_3 > 0.06$, the data set will still remain non-significant, and if $p_1 \text{ or } p_2 > 0.06$ and $p_3 \leq 0.06$, then the combination data set becomes statistically significant. Overall, the statistical probability for the combined data at 24 and 48 hrs (p_3) of the extension model provides a more accurate analysis of finding the effective growth cycle on the scaffold matrix.

The inhibition concentration of MCF-7 cells exposed to the different scaffolds were calculated from the extended probit data (P3) (Table 7) and the cumulative data distribution (Figure 3, Table 7). A correlated probit model was also used to address the binary responses for a developmental toxicity study of foetal weight and mice malformation²⁷. The correlation coefficient values were calculated for (probit P3 data, Log_{10}C in Tables 1 to 6) and the (cumulative data, Log_{10}C in Figure 3, and Table 7). The coefficient values in Table 7 showed the strength of the correlation using the probit data or the cumulative data to calculate the IC_{50} . Compared to the probit data (P3), the cumulative data showed a stronger negative correlation coefficient (-0.99) for the plot of IC_{50} . Thus, this method was considered reliable in this study. The IC_{50} values determined from the distribution data suggests the scaffold systems are more suited for inhibitory assays for MCF-7 cells.

It has been reported that the presence of silica nanoparticles in a mixture helps to increase the interfacial binding ²⁸, and this cross-linking can easily be achieved at room temperature ²⁹. Higher fraction of water or organic solvents in the mixture for fabricating the scaffold systems may reduce the cell viability, and that may affect the overall quality of the assay development. The cell viability of MCF-7 cells exposed to *Terminalia ferdinandiana* Excell (Kakadu plum) fruit and leaf extracted with methanol, water, ethyl acetate, hexane and chloroform were analyzed. The statistical probability values of p_1 , p_2 and p_3 from the data set (Table 8) indicated leaf and fruit extracts from methanol, water, ethyl acetate, hexane and chloroform as statistically relevant using the following additional rules: p_1 or $p_2 \leq 0.06$, $p_3 > 0.06$ are non-statistically significant data sets, and p_1 or $p_2 > 0.06$ and $p_3 \leq 0.06$ as significant data sets. Additional data screened using the set of rules: p_1 or p_2 or $p_3 \leq 0.06$ demonstrated the statistical relevance of the assay type. Subsequently, the assay choices were scaled down to methanolic leaf extract and fruit ethyl acetate extract. *T. ferdinandiana* has high levels of the following bioactive compounds containing : ascorbic acid (vitamin C), gallic acid (also known as 3,4,5-trihydroxybenzoic acid, chlorophyll a and b, ellagic acid, α tocopherol (vitamin E), kaempferol, quercetin, lutein, luteolin, and hesperitin. ³⁰ Recently, the inhibitory activities of Kakadu plum fruits and leaves were tested against several human carcinoma cell lines. ²¹ While some of these molecules are potent cell growth inhibitors, the activities of nanoparticles mixed with plant extracts remain to be investigated. As a follow up study, PEGylated SNP (SNP:PEG) was mixed with leaf methanolic extracts and fruit ethyl acetate extracts. Figure 8 showed the profiled data set of MCF-7 cells exposed to Kakadu plum leaf and fruit extracts from different solvents. The calculated p-values suggest the data from the leaf extracts from methanol, water and hexane, and fruit extract from methanol, water, ethyl acetate, hexane, and chloroform are statistically significant.

The antitumor activity of cisplatin has been known since 1960s and the drug potency has been widely tested in different cell lines, animals and patients. ^{31, 32} The main action of cisplatin is that it inhibits transcription as well as inhibit the DNA replication mechanisms. ³³ It can also interfere with other signal transduction pathways, causing induced apoptosis. ³⁴ Cellular death induced by cisplatin is drug-dose dependent and varies for different cancer cell lines. ^{35, 36} Furthermore, cancer cells can also become cisplatin resistant and a combination-chemotherapy with cisplatin treatment has been suggested as the most effective treatment. ³¹ In this study, a cellular combination treatment using the scaffold matrix/cisplatin dose was used to inhibit cell growth. The cisplatin dose response using the 96-well plate alone indicated an average of 20% cell viability (Figure 4a). Subsequently, 7.5 $\mu\text{g/mL}$ (25 μM) was used as the cellular chemotherapeutic dose. Interestingly, there was a reduction in cell viability to 5%, for the scaffold containing PEGylated SNP (SNP: PEG) substrate (Figure 4b) with a mixture ratio of 10:1. There was no inhibitory activity of cisplatin on MCF-7 cells grown on scaffold

containing Kakadu plum leaf methanol extract mixed with PEGylated SNP (Figure 4c). There was also no inhibitory activity of cisplatin on MCF-7 cells grown on scaffold containing Kakadu plum fruit ethyl acetate extract mixed with PEGylated SNP (Figure 4e). It was noted that the combination treatment of Kakadu/PEGylated SNP induced the cancer cells to be cisplatin resistant. Interestingly, a significant activity with the combination treatment using PEGylated SNP/cisplatin exposed to cancer cells was noted.

Conclusion

Batch-effect is an integral part of scaffold development and affects data processing. It may cause high attrition rates in scaffold-based *in vitro* preclinical studies. The combination basic rules implemented in this study was used to narrow assay choices as well as to identify reproducible data sets that are statistically significant. The chemotherapeutic dose (56 mg/m²) exposed to MCF-7 cells reduced the cell viability to 5% within 24 hours for scaffold containing silica nanoparticles and polyethylene glycol mixtures (SNP: PEG) in a ratio of 10:1, respectively. The combined approach (PEGylated SNP/anticancer drug) may be used intravenously for solid tumor studies involving *in vivo* drug discovery.

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Fig. 1. Effective growth of MCF-7 cells exposed to scaffold system : (A) SNP (B) SNP : PEG (C) SNP : PEG : dendrimer. Proliferation frequency, $f(\%)$: $f(t) = \frac{k_2(t_2)}{k_1(t_1)}$. $k_1 = \frac{1}{t} \ln \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,24hrs}}{y_o}$; $k_2 = \frac{1}{t} \ln \frac{\sum_{i=1}^n (\mu - \bar{s})_{c,48hrs}}{y_o}$. These equations have been explained in equations 10 and 11. The scaffolds were fabricated under the low and high humidity conditions: $\Delta = 37^\circ\text{C}$, 15% humidity and 30°C , 90% humidity prior to seeding MCF-cells at the same concentration (10^3 cells per 100 mL).

Fig.2. Assessment of the standard probit (P1 and P2) and the probit extension models (P3). P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs. The graphs indicate MCF-7 cells data when exposed to the different scaffold systems: (A ,B) SNP (C,D) SNP : PEG (E,F) SNP : PEG : dendrimer, under low and high humidity conditions respectively.

Fig. 3. Inhibition concentration (IC_{50}). The graphs indicate the inhibition concentration require to inhibit at least 50% of the MCF-7 cells in population after 48 hrs. The IC_{50} is the 50% of the highest cumulative data from the distribution, and the inhibition concentration is calculated from the linear fit of the distribution. The different scaffold systems: (A ,B) SNP (C,D) SNP : PEG (E,F) SNP : PEG : dendrimer, under low and high humidity conditions, respectively.

Fig.4. Cell viability of MCF-7 cells after cisplatin dosing (A) on control, 96 well plate (B) on substrate cast of SNP: PEG scaffold (C) on substrate cast of SNP:PEG:KPLM scaffold (D) on substrate cast of SNP:PEG:KPFE scaffold. Relative standard deviation of 5.0% (n=4).

Table 1. Probit analysis and probit extension models : MCF-7 cells exposed to SNP scaffold assembled under low humidity conditions.

1- cell value in column 8															
											P2	P1		P3	
	wt.%	24hrs	48hrs	f	cf	cf inverse	Concentration			Log ₁₀ C	100 % - Column 4	Probit values for Column 12	100% - Column 3	Probit values for column 14	Probit values for column 9
N							X	%							
1	40	40%	30%	0.75	0.75	1.34	1.00	0%	0.4	-0.40	0.70	5.52	0.60	5.25	0
2	35	78%	35%	0.45	1.20	0.83	0.62	38%	0.35	-0.46	0.65	5.39	0.22	4.23	4.69
3	30	87%	46%	0.52	1.73	0.58	0.43	57%	0.3	-0.52	0.54	5.1	0.13	3.87	5.18
4	25	90%	51%	0.56	2.29	0.44	0.33	67%	0.25	-0.60	0.49	4.97	0.10	3.72	5.44
5	20	91%	53%	0.58	2.87	0.35	0.26	74%	0.2	-0.70	0.47	4.92	0.09	3.66	5.64
6	15	91%	54%	0.60	3.47	0.29	0.22	78%	0.15	-0.82	0.46	4.9	0.09	3.66	5.77
7	10	90%	54%	0.60	4.07	0.25	0.18	82%	0.1	-1.00	0.46	4.9	0.10	3.72	5.92
8	5	87%	54%	0.62	4.69	0.21	0.16	84%	0.05	-1.30	0.46	4.9	0.13	3.87	5.99
9	4	85%	52%	0.61	5.30	0.19	0.14	86%	0.04	-1.40	0.48	4.95	0.15	3.96	6.08
10	3	85%	52%	0.61	5.91	0.17	0.13	87%	0.03	-1.52	0.48	4.95	0.15	3.96	6.13
11	2	83%	50%	0.61	6.52	0.15	0.11	89%	0.02	-1.70	0.50	5	0.17	4.05	6.23
12	1	84%	49%	0.58	7.10	0.14	0.11	89%	0.01	-2.00	0.51	5.03	0.16	4.01	6.23

f, proliferation frequency factor, calculated from equation 11; X is the standardised cf inverse value. P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs.

Table 2. Probit analysis and probit extension models : MCF-7 cells exposed to SNP scaffold assembled under high humidity conditions.

1- cell value in column 8											P2	P1		P3	
	SNP (wt.%)	24hrs	48hrs	f	cf	cf inverse	Concentration		Log ₁₀ C	1 - Column 4	Probit values for Column 12	1 - Column 3	Probit values for column 14	Probit values for column 9	
N	Cells						X	%		%		%			
1	40	76%	44%	0.58	0.58	1.72	1.00	0%	0.4	-0.40	0.56	5.15	0.24	4.29	0
2	35	95%	57%	0.60	1.18	0.85	0.49	51%	0.35	-0.46	0.43	4.82	0.05	3.36	5.03
3	30	97%	58%	0.60	1.78	0.56	0.33	67%	0.3	-0.52	0.42	4.8	0.03	3.12	5.44
4	25	97%	60%	0.62	2.40	0.42	0.24	76%	0.25	-0.60	0.40	4.75	0.03	3.12	5.71
5	20	96%	61%	0.64	3.03	0.33	0.19	81%	0.2	-0.70	0.39	4.72	0.04	3.25	5.88
6	15	100%	60%	0.60	3.64	0.27	0.16	84%	0.15	-0.82	0.40	4.75	0.00	0	5.99
7	10	99%	59%	0.60	4.23	0.24	0.14	86%	0.1	-1.00	0.41	4.77	0.01	2.67	6.08
8	5	91%	55%	0.60	4.84	0.21	0.12	88%	0.05	-1.30	0.45	4.87	0.09	3.66	6.18
9	4	89%	53%	0.60	5.43	0.18	0.11	89%	0.04	-1.40	0.47	4.92	0.11	3.77	6.23
10	3	86%	50%	0.58	6.02	0.17	0.10	90%	0.03	-1.52	0.50	5	0.14	3.92	6.28
11	2	86%	51%	0.59	6.61	0.15	0.09	91%	0.02	-1.70	0.49	4.97	0.14	3.92	6.34
12	1	77%	43%	0.56	7.17	0.14	0.08	92%	0.01	-2.00	0.57	5.18	0.23	4.26	6.41

f, proliferation frequency factor, calculated from equation 11; X is the standardised cf inverse value. P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs.

Table 3. Probit analysis and probit extension models : MCF-7 cells exposed to SNP: PEG scaffold assembled under low humidity conditions.

1- cell value in column 8										P2		P1		P3		
N	Ratio . SNP : PEG mixture	24hrs	48hrs	f	cf	cf inverse				Blank	Log ₁₀ C	1 - Column 4	Probit values for Column 12	1 - Column 3	Probit values for column 14	Probit values for column 9
							X	%			%		%			
1	40.00	57%	31%	0.55	0.55	1.82	1.00	0%		1.60	69%	5.50	43%		4.82	0.00
2	17.50	62%	35%	0.57	1.12	0.89	0.49	51%		1.24	65%	5.30	38%		4.69	5.03
3	10.00	63%	39%	0.61	1.74	0.58	0.32	68%		1.00	61%	5.28	37%		4.67	5.47
4	6.25	70%	41%	0.58	2.31	0.43	0.24	76%		0.80	59%	5.23	30%		4.48	5.71
5	4.00	68%	41%	0.60	2.92	0.34	0.19	81%		0.60	59%	5.23	32%		4.53	5.88
6	1.50	74%	42%	0.57	3.49	0.29	0.16	84%		0.18	58%	5.20	26%		4.36	5.99
7	0.67	69%	38%	0.55	4.04	0.25	0.14	86%		-0.18	62%	5.31	31%		4.50	6.08
8	0.25	58%	30%	0.52	4.56	0.22	0.12	88%		-0.60	70%	5.52	42%		4.80	6.18
9	0.16	51%	29%	0.56	5.11	0.20	0.11	89%		-0.80	71%	5.55	49%		4.97	6.18
10	0.10	48%	26%	0.53	5.64	0.18	0.10	90%		-1.00	74%	5.64	52%		5.05	6.28
11	0.06	44%	20%	0.46	6.10	0.16	0.09	91%		-1.24	80%	5.84	56%		5.15	6.34
12	0.03	39%	16%	0.40	6.50	0.15	0.08	92%		-1.60	84%	5.99	61%		5.28	6.41

f, proliferation frequency factor, calculated from equation 11; X is the standardised cf inverse value. P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs.

Table 4. Probit analysis and probit extension models : MCF-7 cells exposed to SNP: PEG scaffold assembled under high humidity conditions.

1- cell value in column 8										P2		P1		P3		
N	Ratio ,SNP : PEG mixture	24hrs	48hrs	f	cf	cf inverse				Blank	Log ₁₀ C	1 - Column 4	Probit values for Column 12	1 - Column 3	Probit values for column 14	Probit values for column 9
							X	%			%		%			
1	40.00	44%	35%	0.01	0.81	1.24	1.00	0%		1.60	65%	5.39	56%		5.15	0
2	17.50	50%	41%	0.03	1.62	0.62	0.50	50%		1.24	59%	5.23	50%		5	5
3	10.00	51%	43%	0.05	2.46	0.41	0.33	67%		1.00	57%	5.18	49%		4.97	5.44
4	6.25	53%	44%	0.08	3.29	0.30	0.25	75%		0.80	56%	5.15	47%		4.92	5.67
5	4.00	53%	44%	0.13	4.11	0.24	0.20	80%		0.60	56%	5.15	47%		4.92	5.84
6	1.50	58%	46%	0.39	4.90	0.20	0.16	84%		0.18	54%	5.1	42%		4.8	5.99
7	0.67	54%	43%	0.81	5.70	0.18	0.14	86%		-0.18	57%	5.18	46%		4.9	6.08
8	0.25	45%	33%	1.81	6.44	0.16	0.13	87%		-0.60	67%	5.44	55%		5.13	6.13
9	0.16	41%	31%	2.59	7.18	0.14	0.11	89%		-0.80	69%	5.5	59%		5.23	6.23
10	0.10	37%	26%	3.75	7.87	0.13	0.10	90%		-1.00	74%	5.64	63%		5.33	6.28
11	0.06	34%	24%	5.89	8.59	0.12	0.09	91%		-1.24	76%	5.71	66%		5.41	6.34
12	0.03	28%	20%	11.29	9.31	0.11	0.09	91%		-1.60	80%	5.84	72%		5.58	6.34

f, proliferation frequency factor, calculated from equation 11; X is the standardised cf inverse value. P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs.

Table 5. Probit analysis and probit extension models : MCF-7 cells exposed to SNP: PEG : (1^o) PAMAM dendrimer scaffold assembled under low humidity conditions.

								1- cell value in column 8	Log ₁₀ C			P2	P1	P3
		24hrs	48hrs	f	cf	cf inverse			Blank	1 - Column 4	Probit values for Column 12	1 - Column 3	Probit values for column 14	Probit values for column 9
N							X	%		%		%		
1	200.00	41%	68%	1.66	1.66	0.60	1.00	0%		2.30	32%	4.53	59%	0
2	87.50	21%	85%	4.14	5.80	0.17	0.29	71%		1.94	15%	3.96	79%	5.55
3	50.00	23%	47%	2.03	7.83	0.13	0.21	79%		1.70	53%	5.08	77%	5.81
4	31.25	49%	81%	1.66	9.49	0.11	0.18	82%		1.49	19%	4.12	51%	5.92
5	20.00	30%	87%	2.90	12.39	0.08	0.13	87%		1.30	13%	3.87	70%	6.13
6	7.50	11%	64%	5.88	18.27	0.05	0.09	91%		0.88	36%	4.64	89%	6.34
7	3.33	15%	69%	4.55	22.82	0.04	0.07	93%		0.52	31%	4.5	85%	6.48
8	1.25	13%	64%	5.02	27.83	0.04	0.06	94%		0.10	36%	4.64	87%	6.55
9	0.80	20%	72%	3.59	31.42	0.03	0.05	95%		-0.10	28%	4.42	80%	6.64
10	0.50	33%	84%	2.55	33.98	0.03	0.05	95%		-0.30	16%	4.01	67%	6.64
11	0.29	6%	61%	10.35	44.32	0.02	0.04	96%		-0.54	39%	4.72	94%	6.75
12	0.13	-4%	49%	-12.78	31.55	0.03	0.05	95%		-0.90	51%	5.03	104%	6.64

f, proliferation frequency factor, calculated from equation 11; X is the standardised cf inverse value. P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs.

Table 6. Probit analysis and probit extension models : MCF-7 cells exposed to SNP: PEG : (1^o) PAMAM dendrimer scaffold assembled under high humidity conditions.

		1- cell value in column 8								Log ₁₀ C	P2		P1	P3
		24hrs	48hrs	factor, f	cf	cf inverse			Blank	1 - Column 4	Probit values for Column 12	1 - Column 3	Probit values for column 14	Probit values for column 9
N	Conc						X	%		%		%		
1	200.00	27%	18%	0.68	0.68	1.46	1.00	0%	2.30	82%	5.92	73%	5.61	0
2	87.50	69%	39%	0.57	1.25	0.80	0.55	45%	1.94	61%	5.28	31%	4.5	4.87
3	50.00	43%	25%	0.58	1.83	0.55	0.38	62%	1.70	75%	5.67	57%	5.18	5.31
4	31.25	24%	21%	0.85	2.68	0.37	0.26	74%	1.49	79%	5.81	76%	5.71	5.64
5	20.00	37%	23%	0.61	3.29	0.30	0.21	79%	1.30	77%	5.74	63%	5.33	5.81
6	7.50	5%	6%	1.39	4.67	0.21	0.15	85%	0.88	94%	6.55	95%	6.64	6.04
7	3.33	5%	11%	2.27	6.94	0.14	0.10	90%	0.52	89%	6.23	95%	6.64	6.28
8	1.25	-6%	8%	-1.24	5.71	0.18	0.12	88%	0.10	92%	6.41	106%	4.01	6.18
9	0.80	13%	21%	1.61	7.32	0.14	0.09	91%	-0.10	79%	5.81	87%	6.13	6.34
10	0.50	30%	29%	0.95	8.27	0.12	0.08	92%	-0.30	71%	5.55	70%	5.52	6.41
11	0.29	43%	35%	0.82	9.09	0.11	0.08	92%	-0.54	65%	5.39	57%	5.18	6.41
12	0.13	51%	41%	0.80	9.88	0.10	0.07	93%	-0.90	59%	5.23	49%	4.97	6.48

f, proliferation frequency factor, calculated from equation 11; X is the standardised cf inverse value. P1 and P2 are the cell viability values transformed to probit values at 24 and 48 hrs. P3 is the probit extension model, obtained using the combined data at 24 and 48 hrs.

Table 7. Profiling MCF-7 cells exposed to single, two- and three-composite scaffolds.

Method	I				II		III	
	Observed	Probit model		Probit extension model	Inhibition concentration (IC ₅₀)		Correlation coefficient	
	N	P1	P2	P3	Probit P3 data	Cumulative data (%) from P3	Probit P3 data	Cumulative data (%) from P3 data
Cell viability studies of MCF-7 cells exposed to the following scaffolds:								
a. SNP								
1. Low humidity: Δ=37 °C, 15 % humidity	12	0.48	0.11	0.05	0.18 wt. %	0.1 wt. %	-0.58	-0.99
2. High humidity: Δ=30 °C, 90 % humidity	12	0.31	0.09	0.06	0.18 wt. %	0.1 wt. %	-0.55	-0.99
b. SNP : PEG								
1. Low humidity: Δ=37 °C, 15 % humidity	12	0.02	0.004	0.02	2.9	0.79	-0.65	-0.99
2. High humidity: Δ=30 °C, 90 % humidity	12	0.01	0.003	0.02	3.3	1.1	-0.65	-0.99
c. SNP: PEG: Dendrimer								
1. Low humidity: Δ=37 °C, 15 % humidity	12	0.85	0.36	0.03	10 mg/mL	4.29 mg/mL	-0.64	-0.99
2. High humidity: Δ=30 °C, 90 % humidity	12	0.92	0.64	0.01	15.8 mg/mL	3.72 mg/mL	-0.68	-0.99

I . probit data : P1 is the probit data for the viability of MCF-7 cells at 24 hrs ; P2 is the probit data for the viability of MCF-7 cells at 48 hrs; P3 is the extension data, obtained from the combined data of P1 and P2. The statistical significance value is the p-value. The statistical significance range, $0 < p < 0.06$ was used to assess the assay performance. Additional basic rules, ($p1$ or $p2 \leq 0.06$, and $p3 \leq 0.06$ are the data sets that are not reproducible but statistically significant. When the following conditions are fulfilled : $p1$ or $p2$ or $p3 \leq 0.06$, data sets are statistically significant and reproducible.

II. Inhibition concentration (IC₅₀): the IC₅₀ was calculated from the plot of probit (probit P3 versus Log₁₀C); the cumulative data was calculated from the actual data (%) before being transformed to P3. The IC₅₀ was calculated from the plot of cumulative distribution data (%) versus Log₁₀C. The IC₅₀ is the 50% of the highest cumulative data from the distribution.

III. Correlation coefficient was used to calculate the statistical relationship between (probit P3 versus Log₁₀C) and (cumulative distribution data (%) versus Log₁₀C).

Table 8. Profiling MCF-7 cells exposed to Kakadu plum plant extracts.

Method	Observed	Probit model		Probit extension model
	N	P ₁	P ₂	P ₃
p-values				
Proliferation studies on well plates only				
a. Kakadu plum leaf extracts				
1. Methanol	8	0.003	2.28E-05	0.06
2. Water	8	0.58	4.36E-05	0.05
3. Ethyl acetate	8	0.02	0.001	0.13
4. Hexane	8	0.12	0.69	0.05
5. Chloroform	8	0.01	0.2	0.08
b. Kakadu plum fruit extracts				
1. Methanol	8	0.65	0.5	0.06
2. Water	8	0.27	0.04	0.06
3. Ethyl acetate	8	0.01	0.09	0.05
4. Hexane	8	0.61	0.009	0.05
5. Chloroform	8	0.8	0.56	0.05

P1 is the probit data for the viability of MCF-7 cells at 24 hrs ; P2 is the probit data for the viability of MCF-7 cells at 48 hrs; P3 is the extension data, obtained from the combined data of P1 and P2. The statistical significance value is the p-value. The statistical significance range, $0 < p < 0.06$ was used to assess the assay performance. Additional basic rules, (p_1 or $p_2 \leq 0.06$, and $p_3 \leq 0.06$ are the data sets that are not reproducible but statistically significant. When the following conditions are fulfilled : p_1 or p_2 or $p_3 \leq 0.06$, data sets are statistically significant and reproducible.

Table 9. Profiling MCF-7 cells exposed to different scaffolds and cisplatin drug (dose, 7.5 µg/mL).

Method	Observe d	Probit model		Probit extensio n model
	N	P ₁	P ₂	P ₃
	p-values			
SNP:PEG:KPLM scaffold				
1. Low humidity: Δ= 37 °C, 15 % humidity	12	0.0008	0.13	0.65
2. High humidity: Δ= 30 °C, 90 % humidity	12	0.01	0.0007	0.04
With cisplatin drug, 7.5 µg/mL				
SNP:PEG scaffold				
High humidity: Δ= 30 °C, 90 % humidity	8	p-values 0.04		
SNP:PEG:KPLM scaffold				
High humidity: Δ= 30 °C, 90 % humidity	8	0.68		
SNP:PEG:KPFE scaffold				
High humidity: Δ= 30 °C, 90 % humidity	8	0.11		

P1 is the probit data for the viability of MCF-7 cells at 24 hrs ; P2 is the probit data for the viability of MCF-7 cells at 48 hrs; P3 is the extension data, obtained from the combined data of P1 and P2. The statistical significance value is the *p*-value. The statistical significance range, $0 < p < 0.06$ was used to assess the assay performance. Additional basic rules, (p_1 or $p_2 \leq 0.06$, and $p_3 \leq 0.06$ are the data sets that are not reproducible but statistically significant. When the following conditions are fulfilled : p_1 or p_2 or $p_3 \leq 0.06$, data sets are statistically significant and reproducible.

Abbreviations used

MCF-7 = Breast cancer cell line

SNP = Silica nanoparticles

MTS = [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

ECM = Extracellular matrix

PEG = Polyethylene glycol

PEGylated SNP = Composite scaffolds derived from SNP : PEG formulation mixtures

1° PAMAM = Polyamidoamine first generation

DMEM = Dulbecco's Modified Eagle Medium

FBS = fetal bovine serum

HBSS = Hanks balanced salt solution

PVC = Polyvinyl chloride

T. ferdinandiana = *Terminalia ferdinandiana* Exell

KPLM = *T. ferdinandiana* leaf methanolic extract

KPFE = *T. ferdinandiana* fruit ethyl acetate extract

IC₅₀ = Inhibitory concentration (Scaffold inhibition concentration to cancer cells)

ANOVA = Analysis of variance