A new cell-sized support for 3D cell cultures based on recombinant spider silk fibers

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Abstract
It is now generally accepted that 2D cultures cannot accurately replicate the rich environment and complex tissue architecture that exists in vivo, and that classically cultured cells tend to lose their original function. Growth of spheroids as opposed to 2D cultures on plastic has now been hailed as an efficient method to produce quantities of high-quality cells for cancer research, drug discovery, neuroscience, and regenerative medicine. We have developed a new recombinant protein that mimics dragline spidersilk and that self-assembles into cell-sized coils. These have high thermal and shelf-life stability and can be readily sterilized and stored for an extended period of time. The fibers are flexible, elastic, and biocompatible and can serve as cell-sized scaffold for the formation of 3D cell spheroids. As a proof of concept, recombinant spidersilk was integrated as a scaffold in spheroids of three cell types: primary rat hepatocytes, human mesenchymal stem cells, and mouse L929 cells. The scaffolds significantly reduced spheroid shrinkage and unlike scaffold-free spheroids, spheroids did not disintegrate over the course of long-term culture. Cells in recombinant spidersilk spheroids showed increased viability, and the cell lines continued to proliferate for longer than control cultures without spidersilk. The spidersilk also supported biological functions. Recombinant spidersilk primary hepatocyte spheroids exhibited 2.7-fold higher levels of adenosine triphosphate (ATP) continued to express and secrete albumin and exhibited significantly higher basal and induced CYP3A activity for at least 6 weeks in culture, while control spheroids without fibers stopped producing albumin after 27 days and CYP3A activity was barely detectable after 44 days. These results indicate that recombinant spidersilk can serve as a useful tool for long-term cell culture of 3D cell spheroids and specifically that primary hepatocytes can remain active in culture for an extended period of time which could be of great use in toxicology testing.

Keywords
3D cell culture, scaffold, spheroids, spidersilk, hepatocytes, 3D model, drug screening model

Introduction
During the last 15 years, it has become increasingly clear that monolayer cell cultures with cells attached to a polystyrene surface, or even to a surface coated with extracellular matrix compounds, do not completely reflect the conditions in the physiological 3D tissue. Cells on plastic dishes are polarized with their receptors concentrated on the surface in contact with the dish. They are also subjected to a number of differences, for example, with respect to permeation of oxygen, nutrients, or toxic compounds compared to the environment in the tissue. Without the native 3D network of interactions with extracellular matrix or with their neighbors, cells tend to de-differentiate.¹⁻³

In consequence, monolayer cultures are often insufficient to provide a representative in vitro model, leading the researchers to perform the expensive in vivo studies, which themselves may also be insufficient, due to the differences between species.

Based on this need, multiple approaches have been developed to grow 3D cell cultures.⁴⁻¹⁰

One approach involves growing cells under conditions that minimize or avoid contact with the plastic surface, for example, in hanging drops. While avoiding the possibility of contact with the plastic surface, the cells tend to have contact with neighboring cells, forming 3D spheroids. However, in the absence of any biological matrix, the strong intracellular contacts lead to the eventual compaction of the spheroids¹¹ and cell death.¹²⁻¹⁶

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Another approach is preparing 3D scaffolds. A wide variety of biological and artificial materials have been used to prepare macroscopic scaffolds, in order to allow cells to form 3D structures within a larger porous material. However, a major disadvantage is that the cells are not homogeneously distributed in the matrix, and in addition, problems in the diffusion of nutrients and oxygen may cause stagnation and have an adverse effect on cell viability and biological functions.

The advantages of both approaches can be combined by using cell-sized scaffolds where the cells adhere to the porous matrix, thereby decreasing the tendency of the spheroid to shrink, while sustaining the 3D structure. In order to ensure an efficient supply of oxygen, the size of the spheroids including the scaffold should not be greater than a few hundred microns.

One area that can particularly benefit from an improved method of 3D cell culture is preclinical toxicity testing. In addition to the ethical desire to reduce the numbers of animal-based experiments, neither rodents nor 2D culture systems can recapitulate the human tumor microenvironment. The alterations in growth conditions and interactions with surrounding cells or cell matrix frequently influence the response of the cells to drugs. These deficiencies probably contribute to the dismal rate of clinical success of drug development. There are similar difficulties in interpreting the preclinical results obtained with anticancer drugs. Despite many attempts to prepare suitable cell lines, the gold standard for assessing drug-induced liver injury (DILI), a useful method for assessing drug toxicity, still employs primary hepatocytes. When seeded under conventional culture conditions, hepatocytes adhere to the plastic surface of the cell culture dish within 4 h and then aggregate into groups of 2–10 cells to re-establish intercellular contacts. Unfortunately, in classical 2D cultures, primary hepatocytes start to lose activity after only a few days, making them applicable only for acute toxicity studies. The solution appears to be hepatocyte spheroids, which have been reported to live longer in culture and maintain function in that they continue to secrete albumin and can be stimulated to express the cytochrome P450 (CYP) genes that are required for detoxification of applied materials. Such spheroids made of cryopreserved hepatocytes were reported to be phenotypically and functionally stable for 2–3 weeks, and in case of freshly produced hepatocytes for even up to 5 weeks in culture, and to maintain endogenous hepatic functions, such as albumin and urea production as well as glycogen storage. Naturally produced spheroids are useful but are soft and somewhat difficult to manipulate. In the absence of any biological matrix, the strong intracellular contacts may lead to the eventual compaction of the spheroids and cell death. Natural spidersilk is a material that combines a toughness comparable to that of steel, with elasticity and versatility. In addition, it is extensible and well tolerated by biological systems. Substrates prepared from natural spidersilk or recombinant spidersilk-based proteins have been shown to facilitate the adherence of cultured cells and support growth of material for tissue engineering.

We have developed a new material, composed of cell-sized coils of fibers made from a recombinant dragline spidersilk protein. The artificial sequence is based on the sequence of Araneus diadematus spidersilk dragline ADF4 protein. The fibers are produced in insect cells, using a baculovirus expression system. Our unique process of self-assembly of fibers inside the cells generates fibers (SpheroSeev) with structural characteristics similar to those of the natural dragline.

The final fibers are highly water-insoluble and can withstand temperatures up to 235°C, allowing them to be sterilized by autoclave incubation. A single recombinant spidersilk fiber coil has a diameter of about 15 µm, which is comparable to the size of a single cell. This makes the fibers very suitable for use as a scaffold for the preparation of cell spheroids. Cells grown in the presence of our spidersilk form spheroids. As proof of concept, we present here the effects of recombinant spidersilk fibers on the formation of cell spheroids, cell viability within the spheroids, and the biological functions of cultured cells. Efficient cellular growth with an extended period of survival and function were seen in three cell types tested: primary rat hepatocytes, human mesenchymal stem cells (hMSC), and mouse L929 cells.

**Materials and methods**

**Preparation of spidersilk fibers**

Recombinant Spidersilk fibers (SpheroSeev) were produced by Seevix Material Sciences Ltd. and expressed in insect cells by a proprietary procedure. To stain spidersilk coils with anti-His-tag antibodies, the insect cells were seen in three cell types tested: primary rat hepatocytes, human mesenchymal stem cells (hMSC), and mouse L929 cells.

**Cells and cell culture conditions**

NCTC clone 929 (L929) cells (ATCC CCL-1) and adipose-derived mesenchymal stem cells (MSC) were purchased from the ATCC. Primary rat hepatocytes were obtained from Kurabo Industries, Japan.

L929 were cultured in MEM-NEAA medium supplemented with 10% horse serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. The hMSCs were cultured in MSC...
Basal Medium (ATCC PSC-500-030) supplemented with MSC Growth Kit and 100 U/ml penicillin-0.1 mg/mL, streptomycin-0.25 µg/ml, and amphotericin B (Biological industries). Rat primary hepatocytes were cultured in Williams E medium (Sigma) supplemented with 10% inactivated FBS (Biological industries), 2 mM L-glutamine (Sigma), 1.72 µM insulin (Lifetech), 0.07 µM transferrin (Lifetech), 0.04 µM selenium (Lifetech), and 0.1 µM dexamethasone (Sigma).

**Preparation of spheroids**

The procedure for preparing the spheroids is summarized in Figure 1. Briefly, primary rat hepatocyte cells were thawed according to the manufacturer’s instructions. L929 cells and hMSCs were dissociated with trypsin-EDTA (Biological Industries), then were neutralized, and counted with trypan blue to assess viability. Seeding stock was prepared by diluting the cells in growth media (control group) or media containing different concentrations of spidersilk. The samples were dispensed into ultra-low attachment (ULA) plates (Nunclon Sphaera, NUNC) and incubated at 37°C, 5% CO₂ to allow spheroids to form. Every 2 days (hepatocytes), or 4 days (cell lines), half the medium in each well was replaced with fresh medium. For albumin measurements, supernatants collected from hepatocyte cultures were stored at −20°C.

**Staining with PI and PI/calcein**

To assess cell viability and spheroid morphology, control and treated spheroids were collected into a 1.5 mL tube, washed twice in PBS (Biological Industries) by allowing the tube to stand for 2 min until spheroids sink, aspirating the medium, and replacing with 0.5 mL fresh PBS. Spheroids were stained with 2 µM calcein-AM (Cayman Chemical) and 50 µg/ml propidium iodide (Sigma) diluted in PBS. Spheroids were incubated with the dyes for 15 min and then images were collected with a FV-1200 confocal microscope, Olympus, Japan. Images were analyzed by ImageJ software.

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**Figure 1.** (a) Typical recombinant spidersilk coils within the insect cells during production process. Staining with mouse monoclonal anti-His-tag antibody and Alexa 594 goat anti-mouse IgG. (b) Spheroid assembly: fibers and cells are mixed and seeded on an ultra-low attachment plate. Over the following days, the cells form aggregates containing trapped fibers and eventually establish cell–cell interactions to make stable spheroids. (c) Spheroid composed of primary rat hepatocytes at day 27 of culture. Recombinant spidersilk fibers integrated into the spheroids can be observed on the margins of the spheroid (indicated by the arrows). (d) Mesenchymal stem cell spheroids stained with thioflavin S: green- beta sheets containing fibers; blue- non-specific staining of cells.
Staining with thioflavin S

Thioflavin S (Merck) is a fluorescent stain that changes the excitation and emission spectra when bound to beta sheet rich structures to give a green color. For thioflavin S staining, spheroids were incubated in PBS supplemented with 25 µM thioflavin S (Merck) for 15 min. Spheroids were washed in PBS and then images were collected with a FV-1200 confocal microscope, Olympus, Japan.

Measurement of ATP

Viability and proliferation of the cells were measured using a CellTiter-Glo 3D Cell Viability Assay (Promega) based on quantitation of the cellular ATP levels, according to the manufacturer’s instructions for use.

Measurement of albumin

Albumin levels were measured in the supernatant collected from hepatocytes by using a rat albumin ELISA quantitation set (Bethyl laboratories) according to the manufacturer’s instructions.

Measurement of CYP3A activity

CYP3A activity was measured using a P450-Glo™ CYP3A4 assay kit (Promega) according to the manufacturer’s instructions for use. For CYP3A induction, spheroids were exposed to 50 µM dexamethasone (Sigma) for 48 h before measurement.

Statistics

The experimental groups were compared with the controls using unpaired two-tailed t-test. The p values are presented for each quantitative experiment. A p value below 0.05 was considered statistically significant.

Results

Preparation of Spidersilk fibers

Spidersilk fibers were prepared using recombinant DNA technology. A protein biomimicking dragline spidersilk protein was expressed in insect cells and self-assembled inside the cells into coils. Figure 1(a) shows the formation of the spidersilk coils in the insect cells.

When spidersilk coils were incubated with different types of cells in ULA plates according to the scheme showed in Figure 1(b), the cells formed aggregates containing trapped fibers and eventually established cell–cell interactions to make stable spheroids with the fibers integrated into the structure (Figure 1(c)). Spidersilk fibers intercalated into spheroids were stained with thioflavin S, a fluorescent dye that changes its excitation and emission spectra when bound to beta sheet rich structures. Spidersilk fibers are rich in beta sheets and therefore fluorescent green when bound to thioflavin S. In contrast, non-specific binding of thioflavin S appears blue. The green fluorescence shown in Figure 1(d) indicates that the spidersilk fibers are present throughout the spheroid and maintain the beta sheet conformation.

Effect of spidersilk on spheroid size, integrity, and viability

Primary rat hepatocytes formed spheroids in the non-adhesive plates even without the addition of spidersilk coils. However, in the presence of spidersilk coils, even after the first day of culture, hepatocytes seeded at 7500 cells with 0.4 ng/cell spidersilk formed spheroids that were 18.5% larger than those without added coils (p < 0.001, unpaired t-test, Figure 2(a)). Spidersilk fiber-free spheroids compacted during culture, and by day 17, their size had decreased to 44% of the start diameter (the size on day 1 of culture). This percentage decrease was similar over a seeding range of 1500–7500 cells/well (data not shown). However, in the presence of spidersilk fibers, the shrinkage was moderated, and on day 17 of culture, the spheroids grown in the presence of spidersilk had lost only 35% of their diameter and were 78% larger than the spidersilk-free controls (p < 0.001, unpaired t-test, Figure 2(b)).

In contrast to the non-proliferating primary hepatocytes, L929 fibroblasts and hMSCs can proliferate in culture under appropriate conditions. L929 spheroids seeded at 5000 cells/well grew in size even without spidersilk (Figure 2(c)). However, in the presence of 0.4 ng spidersilk/cell, the diameter of the spheroids increased still more indicating higher proliferation for a longer period. By day 8, the diameter of spidersilk L929 spheroids was 56% higher than that of spidersilk-free spheroids (p < 0.001, unpaired t-test). In contrast, spheroids formed from hMSCs seeded at 1500 cells/well did not increase in size but did grow in a dose-dependent manner when spidersilk coils were added (Figure 2(d)). By day 14, the diameter of spheroids seeded with 0.6 and 2 ng spidersilk/cell were 3.1- and 5.1-fold, respectively, greater than control spheroids (p < 0.001, unpaired t-test). These spheroids maintained their size for at least 18 more days in culture.

The ability of the spidersilk to increase the stability of the cells over long-term culturing is demonstrated in Figure 3(a). Cryopreserved primary hepatocytes cultured without spidersilk spontaneously formed spheroids that disintegrated into single cells by day 37 of culture, while in
the presence of spidersilk coils, the spheroids remained intact. Staining the spheroids with calcein AM (live cells stain green) and PI (dead cells stain red) indicated that the cells in the spheroids grown with spidersilk appeared green and were therefore viable, while not only were the spheroids smaller without spidersilk, but the cells were already dying after 7 days in culture (presence of red staining, Figure 3(b)). The L929 spheroids were seeded at 5000 cells/well, which generates large spheroids. At days 5 and 7, those grown in the presence of spidersilk coils (Figure 3(c)) were large and not necessarily round. In contrast, the corresponding spheroids without spidersilk were much smaller and classically spherical. On day 5, the spheroids without spidersilk already contained a prominent core of dead cells in the center of the spheroid; while in the presence of spidersilk, there were only individual dead cells dispersed throughout the spheroid. By day 7, a central core of dead cells had also appeared in the spidersilk spheroids but still occupied a smaller percentage of the whole spheroid than those grown in the absence of fibers.

Human mesenchymal stem cell spheroids in the absence of spidersilk looked shrunken (Figure 3(d)), with a significant number of dead cells (as shown by PI staining). In contrast, spheroids in the presence of spidersilk (4 ng/cell) were much larger, with very few dead cells. In addition, the morphology of cells in the spidersilk spheroids was more spread, whereas cells in the spidersilk-free spheroids appeared rounder, which is known to influence the fate of MSCs in the culture.

The intracellular ATP concentration is also a measure of cellular viability and activity. Assays of primary rat hepatocytes spheroids cultured for 18 days revealed that the presence of spidersilk increased the levels of ATP in the spheroids (i.e., number of metabolically active cells) by 68% \((p < 0.001, \text{unpaired } t\text{-test})\). On day 44, the level of ATP was 2.7-fold higher in cells cultured with spidersilk \((p < 0.05, \text{unpaired } t\text{-test})\) (Figure 4(a)). This indicates that the cells grown with the spidersilk remain viable and active for at least 6 weeks in culture, which represents a significant advance over regular cultures of primary hepatocytes.

The addition of spidersilk also improved the intracellular ATP concentration of L929 spheroids, which increased by 37% on day 4 \((p < 0.05, \text{unpaired } t\text{-test})\), and by 62% at day 6 of culture compared to control cultures without fibers \((p < 0.05, \text{unpaired } t\text{-test}, \text{Figure 4(b)})\). The viability of hMSCs spheroids seeded without spidersilk decreased slowly but continuously throughout the experiment. In contrast, spidersilk-containing spheroids continued to proliferate for 24 days and maintained high viability for at least 30 days.
Seeding MSC spheroids with 2 ng spidersilk/cell resulted in higher viability than seeding with 0.6 ng spidersilk/cell. At days 24–30, although the spheroids did not increase in size, the viability of cells grown with 2 ng/cell spidersilk was 7–8-fold higher than those grown without spidersilk ($p < 0.01$, unpaired $t$-test).

**Biological function of rat hepatocytes**

The functionality of hepatocytes can be monitored by the ability to secrete serum proteins, specifically albumin, and by the enzymatic activity of CYP3A, a member of the cytochrome P450 superfamily, the major enzymes involved in drug metabolism.

**Albumin secretion**

The ability of cultured rat hepatocytes to produce albumin was increased by inclusion of spidersilk fibers (Figure 5(a)). At day 14 of culture, albumin secretion by hepatocytes in spidersilk spheroids was 96% higher than in control spheroids and continued for many days after cells grown without spidersilk had essentially ceased to secrete any detectable albumin at all. Albumin production by cells grown with spidersilk was still easily quantifiable up to day 44. The results demonstrate that spidersilk both increased the maximal expression and prolonged the period of expression. The effect was particularly prominent at low cell concentrations.
Functionality of primary hepatocyte spheroids cultured with and without spidersilk coils was assessed by CYP3A activity measurement. In addition to the basal CYP3A activity, cells were also treated with dexamethasone, to induce CYP3A as described previously. As shown in Figure 5(b), both basal and induced CYP3A activity were higher in spheroids cultured with spidersilk compared to those without (basal CYP3A $p < 0.001$, induced CYP3A $p < 0.05$, unpaired t-test). At day 32, basal and induced CYP3A activity was 5- and 16-fold higher (respectively) in spheroids generated with spidersilk (basal CYP3A $p = 0.001$, induced CYP3A $p < 0.001$, unpaired t-test). Notably, after 44 days of culture, CYP3A activity was barely detectable in control spheroids while still easily measured in spidersilk spheroids, reaching a 30-fold higher basal activity and 109-fold higher induced activity (basal CYP3A $p < 0.001$, induced CYP3A $p < 0.005$, unpaired t-test). This indicates that even after extended culture, the hepatocytes grown with spidersilk remained active and suitable for use in toxicity assays.

Discussion

We have produced recombinant spidersilk fibers that are made of a stable, elastic, and versatile natural compound and serve as cell-sized scaffolds. The use of the recombinant spidersilk may combine the advantages of two approaches used in 3D cultures, by reducing undesirable spheroid shrinkage and cell death without the formation of a large-scale supporting matrix that prevents free penetration of chemicals and oxygen. Since the scaffold is comprised of discrete particles, it is homogenously distributed throughout the spheroid.
Cultures of all the cell types described here grew better in the presence of the spidersilk coils. This was reflected in a higher viability and activity (ATP assay) in culture over a longer period of time and improved proliferation of dividing cells (fibroblasts and MSCs). We have a particular interest in the primary hepatocyte cultures, in which albumin production and CYP3A activity remained high for over 6 weeks in culture; cells after 44 days in culture with the spidersilk coils responded to induction with dexamethasone over 100-fold better than cultures of primary hepatocytes cultured without the spidersilk coils. This could be of great use in drug toxicity screening, by extending the time frame available for testing DILI. Significantly, 27% of the drugs withdrawn due to toxicity between 1990–2010 were due to DILI,53 which reflects the failure of animal models to recapitulate human metabolism. The increase in the time window could make it possible to conduct repeat tests and longer-term studies, which are very desirable in this context.29

In this study, we emphasized the properties of this unique scaffold in the context of spheroids; however, we believe that future applications for recombinant spidersilk fibers will extend far beyond this phenomenon. Significantly, spidersilk is known to be biocompatible and is well tolerated when implanted.43,54,55

Recently, in accordance with our results, microfibers hydrolyzed from silkworm and incorporated into bone marrow stem cell spheroids were shown to promote higher cell viability and osteogenic differentiation.56

The results presented here show that naturally folded and spontaneously self-assembled recombinant spidersilk can provide a distinctive structure and architecture, with exceptional flexibility and a vast surface area. We believe that the effects of the recombinant spidersilk fibers on spheroid viability and functionality are related to the dimensions, structure, and mechanical properties of the fibers. The structure, composed of nano-dimension fibrils intertwined to form micro-dimension coils, supports the cells in an ECM-like manner, providing a tissue-like environment. The high porosity of the coils permits better penetration of oxygen, nutrients, and waste removal enabling the culture of larger spheroids, not limited to 100–200 µm. The combination of dimensions, unique mechanical properties of the fibers, and the ability to spontaneously adhere to cells give them the capacity to create a personal scaffold for individual cells.

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