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Review

Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures

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Abstract

Biomedical researchers have become increasingly aware of the limitations of time-honored conventional 2D tissue cell cultures where most tissue cell studies have been carried out. They are now searching for 3D cell culture systems, something between a petri dish and a mouse. It has become apparent that 3D cell culture offers a more realistic micro- and local-environment where the functional properties of cells can be observed and manipulated that is not possible in animals. A newly designer self-assembling peptide scaffolds may provide an ideally alternative system. The important implications of 3D tissue cell cultures for basic cell biology, tumor biology, high-content drug screening, and regenerative medicine and beyond could be profound.

Keywords: Beta-sheet structure; Biological scaffolds; Nanofibers and nanopores; Ionic self-complementarity

Contents

Nearly all tissue cells are embedded in 3-dimension (3D) microenvironment in the body. On the other hand, nearly all tissue cells including most cancer and tumor cells have been studied in 2-dimension (2D) petri dish, 2D multi-well plates or 2D glass slides coated with various substrata. The architecture of the in situ environment of a cell in a living organism is 3D, cells are surrounded by other cells, where many extracellular ligands including many types of collagens, laminin, and other matrix proteins, not only allow attachments between cells and the basal membrane [1–3] but also allow access to oxygen, hormones, and nutrients; removal of waste products and other cell types associated in tissues. The normal 3D environment of cells consists of a complex network of extra-

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cellular matrix nanofibers with nanopores that create various local microenvironments, resembling numerous rooms in a sophisticated architectural building complex in a city as large as London, Tokyo, New York or Shanghai (Fig. 3).

1. 2D or not 2D?

There are several key drawbacks to 2D cell cultures. First, the movements of cells in the 3D environment of a whole organism typically follow a chemical signal or molecular gradient. Molecular gradients play a vital role in biological differentiation, determination of cell fate, organ development, signal transduction, neural information transmission and countless other biological processes [4,5]. However, it is nearly impossible to establish a true 3D gradient in 2D culture.

Second, cells isolated directly from higher organisms frequently alter metabolism and alter their gene expression patterns when in 2D culture. It is clear that cellular structure plays a major role in determining cellular activity, though spatial and temporal extracellular matrix protein and cell receptor interactions that naturally exists in tissues and organs. The cellular membrane structure, the extracellular matrix and basement membrane significantly influences cellular metabolism, via the protein–protein interactions. The adaptation of cells to a 2D petri dish requires significant adjustment of the surviving cell population not only to changes in oxygen, nutrients and extracellular matrix interactions, but also to alter waste disposal.

Third, cells growing in a 2D environment can significantly alter production of their own extracellular matrix proteins and often undergo morphological changes (e.g., an increase in spreading). It is not unlikely that the receptors on cell surface could preferentially cluster on parts of the cell that directly expose to culture media rich in nutrients, growth factors and other extracellular ligands; whereas, the receptors on the cells attached to the surface may have less opportunity for clustering. Thus, the receptors might not be presented in correct orientation and clustering, this would presumably also affect communication between cells.

Moreover, in vitro cultured cells lack key signaling and hormonal agents supplied in the in vivo situation by the circulatory system. Presumably this drawback will also be difficult to address with 3D cell culture systems until more is known about physiological environment of cells.

How realistic is a picture of cell behavior that does not take account of cellular communication, the transport of oxygen, nutrients and toxins, and cellular metabolism in the context of all three dimensions?

2. Do scales matter?

In the last two decades, several biopolymers, including PLLA, PLGA, PLLA-PLGA copolymers and other biomaterials including alginate, agarose, collagen gels, etc, have been developed to culture cells in 3D [6–10]. These culture systems have significantly advanced our understanding of cell-material interactions and fostered a new field of tissue engineering. Attempts have been made to culture cells in 3D using synthetic polymers/copolymers. However, processed synthetic polymers consisting of microfibers ∼10–50 μm in diameter are similar in size to most cells (∼5–30 mm in diameter). Thus, cells attached on microfibers are still in a 2D environment with a curvature depending on the diameter of the microfibers. Therefore, cells attached on microfibers are in fact, in 2D despite the various curvatures associated with the large diameter microfibers. Furthermore, the micropores (∼10–200 mm cross) between the fibers are often ∼1000–10,000-fold greater than the size of bimolecular, which as a consequence can quickly diffuse away, much like a car driving on highways. For a true 3D environment, a scaffold's fibers and pores must be much smaller than the cells. In order to culture tissue cells in a truly 3D microenvironment, the fibers must be significantly smaller than cells

Fig. 1. Scales make a big difference. The trees shown on the left are 20–30 cm in diameter and the distance between the trees is in tens of meters. Animals can not walk through, the trees but can walk between them. On the other hand, grass is about 0.5 cm in diameter. When animals walk in the grass field, they are surrounded by the grass. These trees and grass are made of the same cellulose but at different scales.

so that the cells are surrounded by the scaffold, similar to the extracellular environment and native extracellular matrices [11–13].

Animal-derived biomaterials (e.g., collagen gels, poly-glycosaminoglycans and Matrigel) have been used as an alternative to synthetic scaffolds [14–26]. But while they do have the right scale, they frequently contain residual growth factors, undefined constituents or non-quantified impurities. It is thus very difficult to conduct a completely controlled study using such biomaterials because they vary from lot to lot. This not only makes it difficult to conduct a well-controlled study, but also would pose problems if such scaffolds were ever used to grow tissues for human therapies. Animal-derived biomaterials, e.g., collagen gels, laminin, poly-glycosaminoglycans, materials from basement membranes including Matrigel^{TM}, have been used as an alternative to synthetic scaffolds [14–26]. Although researchers are well aware of its limitation, it is one of the only few choices. Thus,

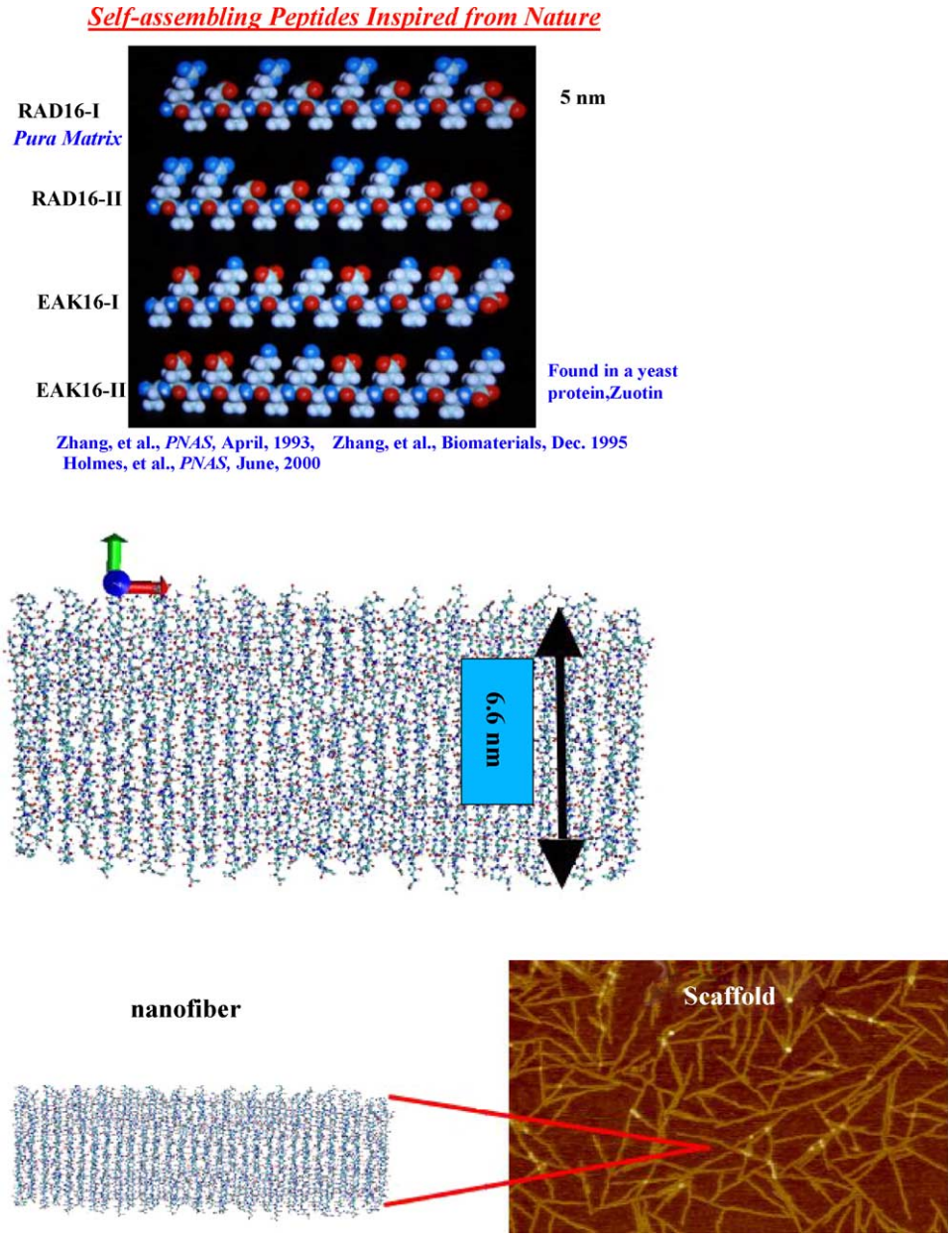

Fig. 2. Molecular models of several self-assembling peptides. (A) Molecular models of RADA16-I, RADA16-II, EAK16-I and EAK16-II. Each molecule is ~5 nm in length with eight alanines on one side and four negative and four positive charge amino acids in an alternating arrangement on the other side. EAK16-II is the first self-assembling peptide that was discovered from a yeast protein, zuotin. Blue = positively charged amine groups on lysine and arginine; red = negatively charged carboxylic acids on aspartic acids and glutamic acids. Light green = hydrophobic alanines. The sequence RADA is similar to a known cell adhesion motif RGD. (B) Molecular model of hundreds self-assembling peptides form a well-ordered nanofiber with defined diameter that is determined by the length of the peptides. (C) Thousands, millions and billions of self-assembling peptides form nanofibers that further form hydrogel, with great than 99% water content.

it not only makes difficult to conduct a well-controlled study, but also would pose problems if such scaffolds are ever used to grow tissues for human therapies.

An ideal three-dimensional culture system is thus required that could be fabricated from a synthetic biological material with defined constituents. In this respect, molecular designer self-assembling peptide scaffolds may be the alternative.

3. Discovery and development of self-assembling peptides

The first molecule of this class of designer self-assembling peptides, EAK16-II, a 16 amino acid peptide, was found as a segment in a yeast protein, zuotin which was originally characterized by binding to left-handed Z-DNA [27]. Zuotin is a 433-residue protein with a domain consisting of 34 amino acid residues (305–339) with alternating alanines and alternating charges of glutamates and lysines with an interesting regularity, AGARAEAEAKAKAE-AEAKAKAESEAKANASAKAD [27]. We subsequently reported a class of biological materials made from self-assembling peptides [28–31]. This biological scaffold consists of greater than 99% water content (peptide content 1–10 mg/ml). They form scaffolds when the peptide solution is exposed to physiological media or salt solution [28–31].

The scaffolds consist of alternating amino acids that contain 50% charged residues [28–31]. These peptides are characterized by their periodic repeats of alternating ionic hydrophilic and hydrophobic amino acids. Thus, these β-sheet structures have distinct polar and non-polar surfaces [28–31]. A number of additional self-assembling peptides including RADA16-I and RADA16-II, in which arginine and aspartic acid residues substitute lysine and glutamate have been designed and characterized for salt-facilitated scaffold formation [28–35]. Stable macroscopic scaffold structures have been produced through the spontaneous self-assembly of aqueous peptide solutions introduced into physiological salt-containing solutions. Several peptide scaffolds have been shown to support cell attachment, enhance cell survival and induce cell differentiation for a variety of mammalian primary and tissue culture cells [31,32,35–38].

4. Structural properties of self-assembling peptides

In general, these self-assembling peptides form stable β-sheet structures in water. They are stable across a broad range of temperature, wide pH ranges in high concentration of denaturing agent urea and guanidium hydrochloride. Although sometimes, they may not form long nanofibers, their β-sheet structure remains largely unaffected [28–31,39].

One of the possible reasons is their unique structure. The alternating alanine residues in the designer self-assembling peptides are similar to silk fibroin such that the alanines pack into inter-digital hydrophobic interactions. The ionic complementary sides have been classified into several moduli (modulus I–IV, etc. and mixtures thereof). This classification scheme is based on the hydrophilic surface of the molecules that have alternating positively and negatively charged amino

Fig. 3. Architecture that mimics three-dimensional cellular architecture? The San Simeon Piccolo Dome in Venice, Italy. Each of the metal rods has a diameter ∼4 cm, 500 times smaller than the size of the dome, a diameter of ∼20 m. Each rod also serves as a construction scaffold for building or repairing the dome that is truly embodied in three dimensions (left panel). When the repair and construction is completed, the scaffold is removed as shown (right panel) [48].

acids, alternating by one residue, two residues, three residues, and so on. For example, charge arrangements for modulus I, modulus II, modulus III, and modulus IV are − + − + − + −+, − − + + − − + +, − − − + + +, and − − − − + + + +, respectively. The charge orientation can also be designed in the reverse orientation, which can yield entirely different molecules. These well-defined sequences allow the peptides to undergo ordered self-assembly, resembling some situations found in well-studied polymer assemblies [28–31,39].

5. EAK16-II and RADA16-I self-assembling peptides

The EAK16-II, AEAEAKAKAEAEAKAK, is the first member in the self-assembling peptide family. EAK16-II is the first peptide to be characterized in detail [28,29] and has also been shown to retain β-sheet structure for extended periods of time (one sample is shown to be stable at room temperature for over 10 years, Zhang, unpublished results) (Fig. 1).

The EAK16-II scaffold was first discovered in the tissue culture media where PC12 cells were used to test for EAK16-II cytotoxicity. The EAK scaffold showed no apparent toxicity, instead, the PC12 cells were found to attach onto the membranous materials where EAK16-II was added. On the other hand, in the dishes where EAK8, a single unit of AEAEAKAK, was used, no scaffold formation was observed [28,30]. The membranous material was examined under SEM that revealed a well-ordered nanofiber structure (Fig. 2). Later using AFM, the well-ordered nanofiber structure is confirmed [39].

We then designed several other peptides altering the amino acid sequences containing RAD motif. RADA16-I, RADARADARADARADARADA, and RAD16-II, RARADADARARADADADA, were studied (Fig. 4). These peptides have motif RAD that is similar to the ubiquitous integrin receptor-binding site RGD [1–3]. While it is not known if these RADA repeats in the scaffold behave similarly to RGD motifs, they have been studied in the context of cell attachment across a number of cells (Table 1) [40]. These peptides form well-ordered nanofibers, similar as EAK16. Interestingly, substitution of G in two locations, RADARGDARADARGDA, or shorten the sequence to 2 units of $(RADA)_2$, RADARADA, not only did result in formation of stable β-sheet, but also no nanofiber self-assembly when studied under the identical conditions as RADA16-I. These observations suggest that the formation stable β-sheet

Table 1
A variety of tissue cells and tissues cultured on peptide scaffolds

Mouse fibroblast	Bovine calf and adult chondrocytes
Chicken embryo fibroblast	Bovine endothelial cells
Chinese hamster ovary	Rat adult liver progenitor cells
Rat pheochromocytoma	Rat cardiac myocytes
Rat neural stem cells	Rat hippocampal neural tissue slice
Mouse embryonic stem cells	Mouse adult neural stem cells
Mouse cerebellum granule cells	Mouse and rat hippocampal cells
Bovine osteoblasts	Hamster pancreas cells
Human cervical carcinoma	Human osteosarcoma
Human hepato-cellular carcinoma	Human neuroblastoma
Human embryonic kidney	Human foreskin fibroblast
Human epidermal keratinocytes	Human neural stem cells

These cells include stable cell lines, primary isolated cells from animals, progenitor and stem cells [40].

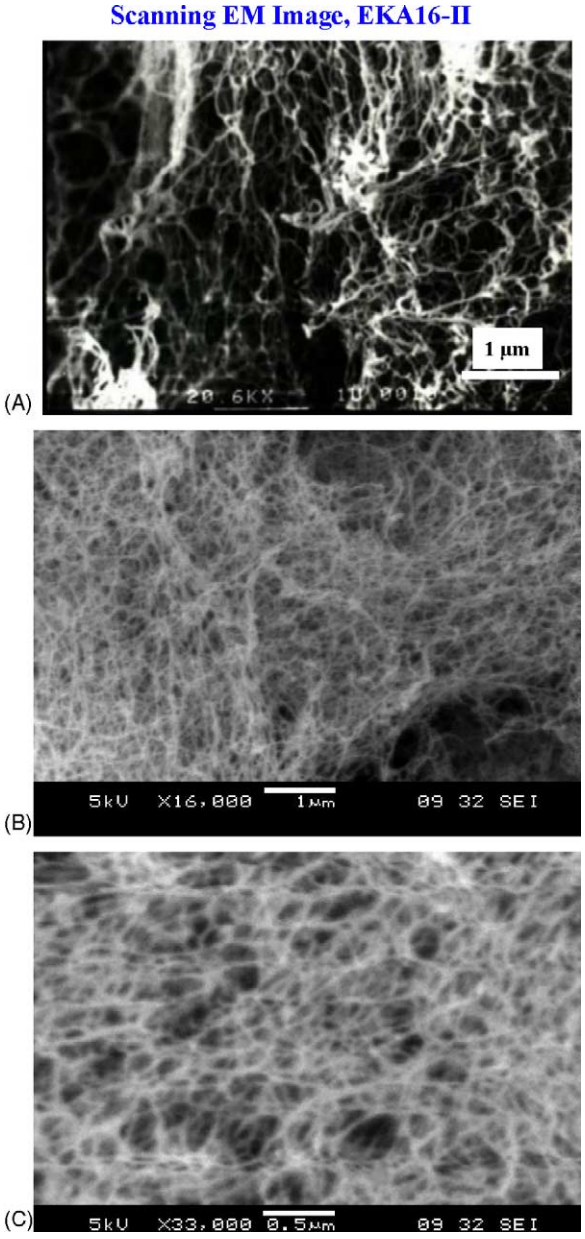

Fig. 4. SEM images of self-assembling peptides. (A) EAK16-II, the first peptide discovered from yeast protein, zuotin; (B) RADA16-I (16,000× magnifications); (C) RADA16-I (33,000× magnifications). These peptides all form nanofiber scaffolds with nanopores (average 5–200 nm). It is worth to point out the nanopores may allow small molecular drugs (1–2 nm) and proteins (2–10 nm) to diffuse in the scaffolds slowly. This is in sharp contract of many other biopolymer microfiber materials where the pores are also microns that drugs and proteins diffuses rather quickly.

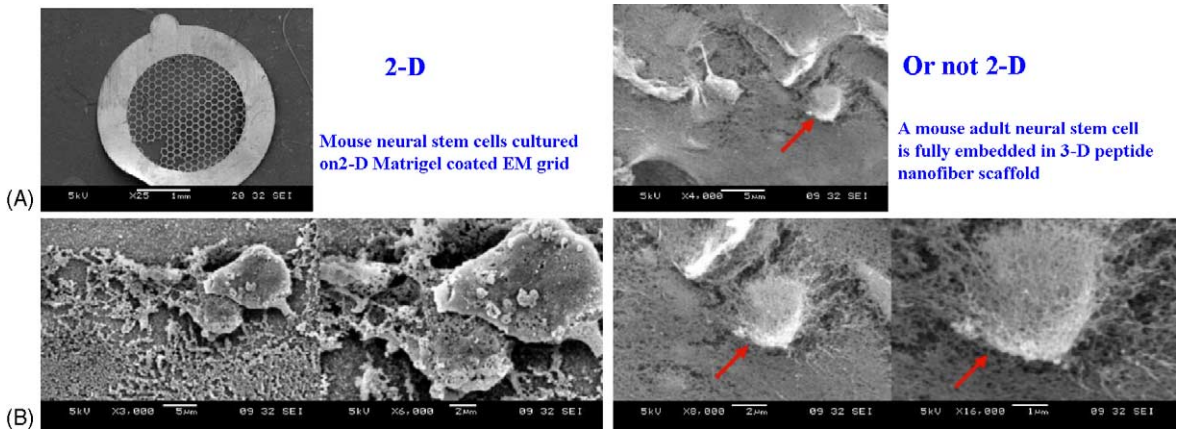

Fig. 5. SEM images of cells on 2D and 3D cell cultures. (A) Cells are cultured on EM grid coated with Matrigel on three different magnifications. Here, only part of the cell surfaces is attached to the rigid surface that may induce adhesion receptors clustering on the attached site. The other side of the cell surfaces is exposed to media where the growth factors, nutrients directly exposed to cells with high concentration through liquid convection. (B) Cell embedded in peptide scaffold in a true 3D manner in different magnifications). The arrows point the same location in all three frames. The cell intimately interact with the nanofiber scaffold where all sides of the cell experience similar adhesion and media exposure.

is important not only for nanofiber but also for scaffold formation (Figs. 4 and 5).

6. A new mode of cell culture

We demonstrated that peptides, made from natural amino acids, undergo self-assembly into well-ordered nanofibers and scaffolds, often ∼10 nm in diameter with pores between 5 and 200 nm. These peptides can be chemically synthesized, designed to incorporate specific ligands, including ECM ligands [1–3] for cell receptors, purified to homogeneity, and manufactured readily in large quantities. Their assembly into nanofibers can be controlled at physiological pH simply by altering NaCl or KCl concentration. Because the resulting nanofibers are 1000-fold smaller than synthetic polymer microfibers, they surround cells in a manner similar to extracellular matrix. Moreover, biomolecules in such a nanoscale environment diffuse slowly and are likely to establish a local molecular gradient.

Using the nanofiber system, every ingredient of the scaffold can be defined, just as in a two dimensional petri dish; the only difference is that cells now reside in a three-dimensional environment where the extracellular matrix receptors on the cell surface can bind to the ligands on the peptide scaffold. Cells can now behave and migrate in a truly three-dimensional manner. Beyond the petri dish, higher tissue architectures with multiple cell types, rather than monolayers, can also be constructed for tissues using the 3D self-assembling peptide scaffolds.

7. Designer peptide scaffolds

In order to fabricate designer peptide scaffolds, it is crucial to understand the finest detail of peptide and protein structures, their influence on the nanofiber structural formation and stability. Since there is a vast array of possibilities to form countless structures, a firm understanding of all available amino acids, their properties, the peptide and protein secondary structures is an absolute prerequisite for further advance fabrication of peptide and protein materials [41,42]. We are moving in that direction and will further accelerate new scaffold development [43–45]. Our results show high level of mouse neural stem cells differentiation toward both neuronal and glial phenotypes in the designer scaffolds in vitro serum-free condition. These results are similar to those with Matrigel, a natural extract considered as the most effective and standard cell-free substrate for neural stem cells culture and differentiation. In the designer peptide scaffolds with functional motifs, not only mouse neural stem cell survival has been significantly improved, but it also enhanced their differentiation, when compared to the self-assembling peptides.

Designer peptide scaffolds so far used in diverse cell and tissue systems from a variety of sources demonstrated a promising prospect in further improvement for specific needs since tissues are known to reside in different microenvironments. The designer peptide scaffolds used thus far are general peptide nanofiber scaffolds and not tailor-made for specific tissue environment. We produced designer peptide scaffolds and showed that these designer peptide scaffolds incorporating specific functional motifs performed as superior scaffolds in specific applications. These designer scaffolds may not only create a fine-tuned microenvironment for 3D tissue cell cultures, but also may enhance cell-materials interactions, cell proliferation, migration, differentiation and performing their biological function. The ultimate goal is to produce designer peptide scaffolds for particular tumor tissue culture as well as for regenerative and reparative medical therapies.

8. Beyond 3D cell cultures for cancer biology studies

Researchers in neuroscience have a strong desire to study neural cell behaviors in 3D and to fully understand their connections and information transmission [47]. Beyond 3D cell culture, since the building blocks of this class of designer peptide scaffolds are natural L-amino acids, the RADA16 has been shown not to elicit noticeable immune response, nor inflammatory reactions in animals [29,46], the degraded products are can be reused by the body, they may also be useful as a bio-reabsorbable scaffold for neural repair and neuroengineering to alleviate and to treat a number of neuro-trauma and neuro-degeneration diseases.

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