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# 3D compartmented model to study the neurite-related toxicity of Aβ aggregates included in collagen gels of adaptable porosity

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ABSTRACT

Insoluble deposits of β-amyloid (Aβ) are associated to neurodegenerative pathologies, in particular Alzheimer's Disease (AD). The toxicity of synthetic amyloid-like peptides has been largely demonstrated and shown to depend upon their aggregation state. However, standard 2D cell culture conditions are not well suited to study the role of the close vicinity of Aβ aggregates and growing neurites in the degenerative process. Here, we have designed a compartmented set-up where model neural cells are differentiated on the surface of Aβ-containing collagen matrices. The average pore size can be modulated, from below 0.2 μm to more than 0.5 μm by simple treatment with collagenase, to respectively hamper or permit neurite outgrowth towards the depth of the matrix. Dense Aβ aggregates (Congo red and ThT-positive) were obtained inside the collagen matrix with a homogeneous distribution and dimensions similar to those observed in post-mortem brain slices from Alzheimer's patients. The aggregates are not toxic to cells when the pore size is small, in spite of relatively high concentrations of 0.05–0.62 mg of peptide per gram of collagen (equivalent to 11.3–113 μM). In contrast, on Aβ-containing matrices with large pores, massive neural death is observed when the cells are seeded in the same conditions. It is the first time to our knowledge that Aβ aggregates with a typical morphology of dense plaques are obtained within a porous biomimetic matrix, and are shown to be toxic only when accessible to differentiating cells.

**Statement of Significance**

Insoluble deposits of β-amyloid (Aβ) are associated to neurodegenerative pathologies, in particular Alzheimer's Disease (AD). In this study, we have formed Aβ aggregates directly inside a biomimetic collagen matrix loaded with growth factors to induce the differentiation of PC12 or SH-SY6Y cells. For the first time, we show that when the contact between cells and Aβ aggregates is allowed by opening up the matrix porosity, the close vicinity with aggregates induces neurite dystrophy. The compartmented 3D culture model developed and used in this study is a valuable tool to study the cytotoxicity of preformed dense Aβ aggregates and proves that contact between the aggregates and neurons is required to induce neurodegenerative processes.



## 1. Introduction

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a progressive neurodegenerative disease. Typical neuropathological features of AD include the presence of extracellular senile plaques, intraneuronal neurofibrilllary tangles as well as loss of synapses and neurons in the cortex and hippocampus of affected individuals [1,2].

Senile plaques are composed mainly of β-amyloid (Aβ) peptides which derive from proteolysis by β- and γ-secretase of a large membrane precursor protein, the amyloid precursor protein (APP). The peptides released in the extracellular space aggregate spontaneously due to their physicochemical properties and form complexes of varying size from small oligomers to insoluble fibrils. The most toxic and amyloidogenic are $A\beta_{1-40}$ and $A\beta_{1-42}$ which

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aggregate in the brain forming senile plaques. They are detected postmortem with a congo red or thioflavin (S or T) staining, which bind specifically to β-pleated sheets. The presence of fibrillar Aβ aggregates induces abnormal activation of adhesion signaling pathways, neuritic dystrophy, and synaptic loss [3,4]. Moreover, some authors illustrate neuronal processes exhibiting loops and sharp turns close to fibrillar Aβ deposits [4,5]. However, more recently, experimental evidence has demonstrated that soluble Aβ monomers or small oligomers, both extracellular and intracellular [6–10], play an important role in AD pathology. To date, most *in vitro* studies consist in treating differentiated cells with a suspension of Aβ aggregates formed in culture medium [4,11–16]. In this case, the whole cell is treated (body and processes) and the peptide aggregation state is often not well characterized. Yet, to study *in vitro* the neurotoxicity of Aβ aggregates specifically, it is important to control the physico-chemical state of Aβ species. Moreover, *in vivo*, Aβ aggregates are primarily located to the grey matter, and therefore only neuritic extensions are in proximity to the senile plaques. Compartmented culture of neural cells proved a valuable tool to investigate the effects of distal assaults on neurites in a variety of neurodegenerative pathologies. Campenot chambers [12,17,18], trans-well [19] and microfluidics devices [20–22] can be used to locally treat the axonal/distal compartment and investigate the cell response. However, the importance of the cell microenvironment in terms of adhesion sites, topology and rigidity has been pointed out in the recent years [23,24] prompting interest for 3D scaffolds, for instance included inside microfluidic chips [25].

We have previously developed a 3D cell culture set-up, based on a porous collagen matrix, well adapted to the adhesion and differentiation of neural cells lines [26]. Type I collagen is not a major component of the brain ECM, which is essentially comprised of hylauronan. However, *in vitro*, collagen can be readily processed into highly hydrated fibrous matrices of controlled porosity, suited as 3D cell culture supports. Moreover, neural progenitor cells embedded in a 3D collagen gel were shown to retain the ability to differentiate into neurons that form functional synapses [27,28]. Such matrices can easily be functionalized with growth factors, either simply impregnated and released, or covalently attached to collagen. This compartmented set-up was designed for the differentiation of neural cells with the cell bodies maintained at the surface of the matrix, while only neurites could grow into the depth of the porous fibrillar network. In this configuration, the growth factors are included in the matrix, and not added to the culture medium, to elicit neurite outgrowth towards the gel depth owing to a chemoattractive effect [29–32]. Another advantage of this set-up is the possibility of direct observation in real-time by confocal microscopy thanks to the controlled thickness and transparency of the matrix.

The aim of the present study was to investigate the specific effects of neurite – Aβ aggregates proximity on the fate of neural cells in this 3D compartmented geometry. Direct formation of Aβ amyloid aggregates inside the matrix was possible because both components, Aβ peptide and collagen monomers, are soluble in acidic conditions and form fibrillar assemblies when the pH is brought to neutrality. We first studied the formation and physical characteristics of the Aβ aggregates embedded in the collagen matrices and their spatial distribution. These Aβ-loaded matrices were then used as 3D substrates with controlled porosity for the culture of two types of model neural cells, PC12 and SH-SY5Y. These cells were often used to study neurite outgrowth [33] and synaptic plasticity [34] and to investigate the mechanisms underlying neurodegeneration in AD [35,36]. On a matrix with small pores, the presence of aggregates does not affect cell viability and neuritic processes phenotype. Finally, we report on the high toxicity of the amyloid aggregates unmasked by a moderate treatment of the matrix with collagenase to open up the surface porosity and permit neurite in-depth outgrowth.

## 2. Materials and methods

### 2.1. Scaffold preparation

#### 2.1.1. Collagen extraction and purification

Type I collagen was extracted and purified from the tails of young Wistar rats following a protocol previously described [37]. Briefly, tendons were excised and washed with phosphate-buffered saline (PBS) to remove cells and traces of blood. Tissues were then soaked in a 4 M NaCl solution to lyse remaining cells and precipitate some of the high-molecular-weight proteins, then rinsed and solubilized in 500 mM acetic acid. The solution was clarified by filtration and centrifugation (21,000×*g* for 2 h). Collagen was then precipitated in 600 mM NaCl, centrifuged (6000×*g* for 45 min at 10 °C) and finally solubilized in 500 mM acetic acid. The solutions were dialyzed (1:10 vol.) at least twice against 500 mM acetic acid to equilibrate the solvent and centrifuged (21,000×*g* for 4 h at 15 °C) to remove remaining impurities or aggregates. A collagen solution at 45 mg/ml was prepared by reverse dialysis against 75 g/l polyethylene glycol (20,000 kDa, Fluka) dissolved in 500 mM acetic acid. Collagen concentration was determined from the acidic solution before fibrillogenesis by assessing the amount of hydroxyproline [38].

#### 2.1.2. Hydrogel scaffold formation, coprecipitation of collagen and Aβ

Amyloid peptide $A\beta_{1-42}$ (referred to as Aβ in what follows) (mouse, rat), sequence: DAEFGHDSGFEVRHQKLVFFAEDVGSNK GAIIGLMVGGVVIA, $M_W = 4418.3\ g\ mol^{-1}$, was purchased from *Polypeptide* (Strasbourg, France). A 2.5 mg/ml stock solution of Aβ (received dry under Argon) was prepared by dissolving 1 mg of the lyophilized peptide in 400 µl Trifluoroacetic acid (TFA) 0.1%, and stored at −80 °C. This stock solution was then diluted if necessary and 15 µl of the dilute solutions were added to 60 mg of collagen in solution (*ca.* 45 mg/ml) to prepare mixtures with 0.05, 0.25 or 0.62 mg of Aβ per gram of collagen. Thorough mixing of the collagen and Aβ acidic solutions was achieved by repeated cycles of mixing and centrifugation. For control gels without Aβ, TFA 0.1% was added to dilute the initial collagen solution accordingly. To facilitate further handling of the matrices, glass coverslips were modified by covalently grafting a collagen monolayer, following the procedure described previously [26]. A drop of the viscous liquid containing collagen and Aβ was then deposited onto a collagen-grafted coverslip placed in a 50 µm-thick Teflon mould closed with a lid and then submitted to ammonia vapors for 3 days. The rise in pH caused by the diffusion of ammonia and subsequent neutralization of the acidic solution induces the formation of an elastic fibrillar gel. The samples were then rinsed by immersing the mould in 0.25× PBS (Phosphate buffer saline diluted ¼ with distilled water) for 12 h. Matrices were kept in a wet atmosphere at 4 °C before use. All manipulations were done in sterile conditions.

For control treatments of cells with Aβ, 20.8 µg of the peptide dissolved in TFA were mixed with culture medium (1 ml) to reach a final concentration of 10 µM and maintained for 3 days at 37 °C before being added to culture wells.

#### 2.1.3. Preparation of conditioned medium and evaluation of toxicity

To directly evaluate the effects on differentiated cells of soluble forms of the peptide potentially released from the matrices containing Aβ aggregates, we prepared a so-called "conditioned medium" as follows. Standard or differentiation culture medium was incubated with Aβ-containing collagen matrices at 37 °C in the