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Metabolite amyloid-like fibrils interact with model membranes†

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Amyloid-like structure formation by various metabolites represents a significant extension of the amyloidogenic building block family. Similar to protein amyloids, metabolite amyloids induce apoptotic toxicity, a process that was linked to membrane association. Here, we demonstrate that metabolite amyloids interact with model membranes and study the mechanism by molecular dynamics.

Amyloidogenic proteins consist of a large group of building blocks that self-assemble into ordered fibrils, showing common structural, biochemical and biophysical features.[1–3] Moreover, the accumulation of these protein assemblies was found to be a hallmark of several major human neurodegenerative diseases.[4] A recent extension to the general amyloid hypothesis was based on the demonstration of the self-assembly of single metabolites into ordered amyloid-like fibrils. Similar to proteinaceous amyloids, the metabolite supramolecular assemblies could bind to indicative dyes, showed a typical fibrillary morphology and triggered cytotoxicity[5–8] *via* an apoptotic mechanism.[6,7] Interestingly, these metabolites were found to accumulate in inborn error of metabolism (IEM) patients, due to a lack of the enzyme catalysing their turnover. Our understanding of the pathological mechanisms that cause the diversified IEM symptoms, which include mental retardation, behavioural abnormalities and seizures, is currently very limited.[9,10] The recently suggested association between neurodegenerative diseases and genetic metabolic disorders *via* the formation of amyloid entities,[5–8] may allow for a better understanding of the pathological mechanisms underlying IEM disorders, and thus may lead to the development of innovative treatments.

As discussed, we previously presented the ability of metabolite amyloid assemblies to induce apoptotic cell death,[6,7] as observed for protein amyloid structures.[11,12] Several possible mechanisms by which this apoptosis effect is triggered have been proposed.[13] In the case of amyloid diseases, interaction of the amyloidogenic assemblies with the cell membrane is considered the prevalent course of toxicity.[14] Similarly, the phenylalanine amino acid also presented an ability to interact with model membrane systems.[15,16] Thus, we were interested in investigating the possible interaction between metabolite amyloid assemblies and model membranes, which may provide further mechanistic insight into their cytotoxicity.

For this purpose, we used the highly-characterized chromatic biomimetic membrane system containing phospholipids and polydiacetylene (PDA). PDA is a lipid-like polymer, which forms vesicular bilayer structures that mimic membrane surfaces. Moreover, PDA exhibits a colorimetric response, namely rapid blue-red fluorescent chromatic response (FCR) transformations, which is induced by external species,[17,18] such as amyloidal assemblies produced by the IAPP polypeptide.[19] In addition to the PDA, two combinations of vesicular bilayer phospholipid compositions were tested, one containing only the phosphatidylcholine phospholipid (PC), a major component of cell membranes, and the other comprising a combination between PC and phosphatidylserine (PS), which is known for its important role in cell cycle signalling, specifically in case of apoptosis.[20] Here, we examined the interaction between these model membrane systems and the tryptophan, tyrosine and adenine amyloid-like assemblies, which accumulate in hypertryptophanemia and hartnup disease, tyrosinemia and adenine phosphoribosyltransferase deficiency, respectively.[9,10] As a control, we used the alanine amino acid, which does not form assemblies and does not trigger cytotoxicity under similar conditions, as was previously established.[5–7] The metabolites self-assembly process

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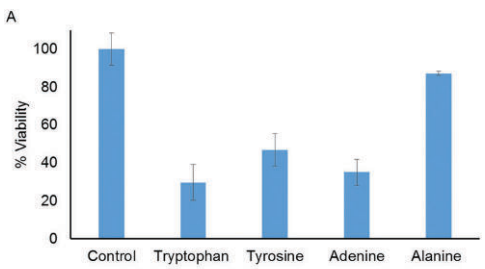
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has been previously described.[6,7] Shortly, metabolites were dissolved in PBS or cell medium, heated to 90° to ascertain the monomeric state, followed by gradual cooling of the solution, resulting in the formation of metabolite amyloid-like fibrils (Fig. S1A–C, ESI†). The concentration of the metabolites used here were previously shown to decrease the viability of neuronal cultured cells to less than 50%,[6,7] which may indicate a similar level of metabolite amyloid fibrils formation. Thus, these metabolites concentrations, which result in a comparable decrease in cell viability, were further explored.

First, the cytotoxic effect of the metabolite assemblies towards human embryonic kidney (HEK)-293 cells was demonstrated (Fig. 1A), resulting in a decrease of cell viability to 30–50%. Next, following a 24 h incubation of the metabolite assemblies or the alanine control with the phospholipid vesicles, the FCR of the system was measured. Using this cell membrane model system, we were able to detect high FCR, implying a strong interaction of the membrane with tryptophan and tyrosine assemblies (Fig. 1B), compared to the alanine that did not cause a FCR effect. Adenine assemblies interacted with both lipid vesicular composition tested, but to a lesser extent.

In order to learn more about the nature of the interaction between the metabolite assemblies and the membrane model, fluorescence quenching experiments were performed.[19,21] NBD-PE [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn-glycero*-3-phosphoethanolamine triethylammonium salt] was incorporated into the phospholipid vesicles, and the effect of the interaction between the membrane and the metabolite assemblies on the fluorescence quenching of NBD-PE was examined. In general, fluorescence quenching of the NBD label results



B

	PS/PC (%FCR)	SD (%)	PC (%FCR)	SD (%)
Tryptophan	10.5	1.0	19	2.1
Tyrosine	20.2	1.2	26.3	0.8
Adenine	5.5	1.5	5.2	0.2
Alanine	-0.2	1.2	-2.3	1.4

Fig. 1 The cytotoxic effect and fluorescent chromatic response (FCR) of metabolite assemblies. Tryptophan (4 mg ml^{-1}), tyrosine (4 mg ml^{-1}) and adenine (2 mg ml^{-1}) metabolite assemblies' solutions were examined. Alanine solution was examined as a control. The results represent three biological repeats. (A) Cytotoxic effect of metabolite assemblies, as determined by MTT assay. HEK-293 cells were incubated with metabolite assemblies and alanine solutions (10 mg ml^{-1}), or medium without any metabolite as a control, for 24 h. The data are presented as mean ± SD. (B) Metabolite assemblies and alanine (4 mg ml^{-1}) solution's interaction with two membrane model systems, PC and a combination of PC with PS, as detected by FCR after 24 h incubation.

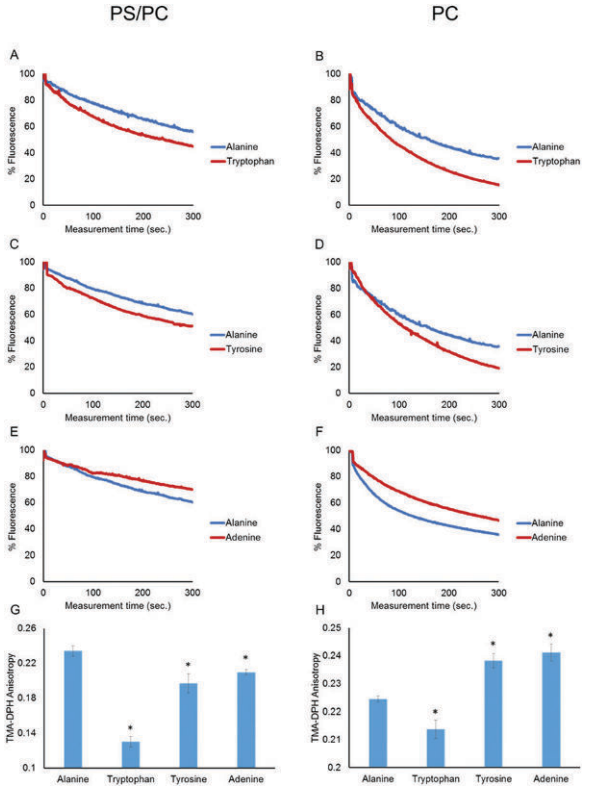


Fig. 2 Metabolite assemblies' interaction with membrane model systems. Tryptophan (4 mg ml^{-1}), tyrosine (4 mg ml^{-1}) and adenine (2 mg ml^{-1}) metabolite assemblies' solutions were examined. Alanine (4 mg ml^{-1}) solution was examined as a control. Two phospholipid compositions of membrane model systems were examined, PC and a combination of PC with PS. (A–F) Quenching of NBD fluorescence as measured after a 24 h incubation with metabolite assemblies', or alanine solution as a control, following an addition of dithionite quencher. The *Y*-axes present the fluorescence values as a percentage of the initial emission reading. (G and H) Fluorescence anisotropy of TMA-DPH, incubated for 24 h with metabolite assemblies' solutions, or alanine solution as a control (*$p < 0.001$ compared to alanine).

from bilayer penetration by membrane-reactive aggregates, while reduced quenching arises from shielding of the NBD dye by the vesicle-bound assemblies. As in the colorimetric vesicles assay, the metabolite assemblies and the alanine control were incubated with the NBD-PE containing vesicles for 24 hours, after which fluorescence quenching measurements were performed. Alanine, used as a negative control, resulted in a similar effect as the untreated control (Fig. S2A and B, ESI†). Both tryptophan and tyrosine assemblies presented strong fluorescence quenching, compared to alanine, in both lipid compositions, suggesting their ability to penetrate the bilayer membrane models (Fig. 2A–D).

Interestingly, adenine assemblies caused a reduction in the quenching compared to the alanine control (Fig. 2E and F). This indicates the ability of adenine assemblies to associate with the model membrane vesicles. The binding of the adenine structures to the lipid part of the membrane model, rather than to the PDA part, may explain the lower FCR response, compared to the significant reduction in the quenching signal.

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To gain further insight into the metabolites interaction with the model membrane systems, the fluorescence anisotropy of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), a common sensitive probe which reacts to the dynamics of its lipid environment, was measured.[22,23] As in the FCR and quenching assays, the fluorescence anisotropy was measured after a 24 h incubation. Alanine presented same effect as the untreated control (Fig. S2C and D, ESI†) and thus served as a negative control. The tryptophan assemblies induced a significant decrease in TMA-DPH fluorescence anisotropy in both phospholipid compositions (Fig. 2G and H), indicating a higher lipid mobility around the probe and an increase in membrane fluidity. Together with the FCR and quenching assays, these results imply the ability of tryptophan assemblies to fully penetrate the model membrane system. Both tyrosine and adenine assemblies induced a significant decrease in TMA-DPH fluorescence anisotropy when tested with the PS/PC lipid composition (Fig. 2G), while in the PC lipid composition, an increase in the fluorescence was observed (Fig. 2H). Overall, in the case of tyrosine amyloid-like fibrils, the results indicate a penetration ability of the assemblies. In the PC lipid composition the structures might be lodged between the lipids, increasing the membrane rigidity. In contrast, a better interaction with the PS/PC composition may allow for a deeper penetration of the tyrosine fibrils to the membrane, resulting in increased membrane fluidity. In the case of adenine amyloid-like fibrils, the assemblies appear to interact with the outer side of the membrane, resulting in an increase in membrane rigidity, while better interaction with the PS/PC composition might allow for a similarly slight increase in membrane fluidity.

To examine the mode of interaction of each metabolite assemblies with the membrane, atomistic molecular dynamics (MD) simulations were performed. Known bulk crystals structures of the metabolites were used, as was previously performed,[7] to model metabolites assemblies and their coupling with membranes. To determine which orientation of each metabolite assembly allows for the best interaction with the membrane, three facets (A–C) of tryptophan, tyrosine and adenine structures were separately coupled with the membrane. In facet A of the tryptophan and tyrosine assemblies, the head groups were in direct contact with the membrane, while in facets B and C the crystals were positioned perpendicular to that of facet A with respect to the membrane. Due to weak coupling in the tryptophan systems, an additional simulation was performed with a system of a half-integral number of bilayers, so that a zwitterion layer was in direct contact with the membrane (zwitterion). The zwitterion layer of the tyrosine structures in direct contact with the membrane was also simulated. In the adenine assemblies, facet A of the crystal was parallel to the membrane, whereas in facets B and C, the planes of the adenine molecules adopted an angle relative to the membrane. Each crystal facet was placed close to a membrane consisting of either only PC phospholipids or 90% PC and 10% PS phospholipids. Furthermore, contact area and interaction energies were calculated and normalized to the contact area of each system.

By observing the trajectories and computing normalized interaction energies, the most stable orientations of each assembly with respect to the membrane were identified (Fig. 3A–C and Fig. S3–S5, ESI†). The tryptophan crystal showed the most favourable interactions with the zwitterion in direct contact with the phospholipid membrane (Fig. 3A and Fig. S3, ESI†), tyrosine showed the most favourable interaction with the polar head groups in direct contact with the membrane (Fig. 3B and Fig. S4, ESI†), and adenine showed the most favourable contact when the plane of the crystalline molecules was parallel to that of the membrane (Fig. 3C and Fig. S5, ESI†). The other orientations showed an unstable configuration. Furthermore, the corresponding interaction energy per contact area was calculated (Fig. 3D). The interaction energy largely corresponded to the FCR experiments, when comparing the association of the individual metabolite crystalline systems with the two membranes. Since the MD simulations data cannot be normalized to the concentration, the comparison between the different metabolites is limited. The tryptophan assemblies showed a stronger binding to the PC phospholipid membrane as compared to a PS/PC membrane, at a ratio similar to that observed in the FCR experiments. The adenine crystalline systems bounded equally to both membranes, as also observed in the FCR analysis. However, in the case of tyrosine, the interaction energies of the crystalline systems are the same for both PC and PS/PC phospholipid membrane, although showing a higher FCR effect for the PC phospholipid. Smaller differences detected by the FCR analysis might require considerable time scales, and probably also more precise force fields, to be identified using MD simulations.

The most stable configuration for the tryptophan structures was driven by coulombic and hydrogen bonding interactions. Due to the bulky apolar head group of the tryptophan molecule, the tryptophan assemblies had maximum interactions with the zwitterion when contacting the membrane. When the apolar head group was in direct contact with the membrane, the structures torqued and changed orientation, thus resembling

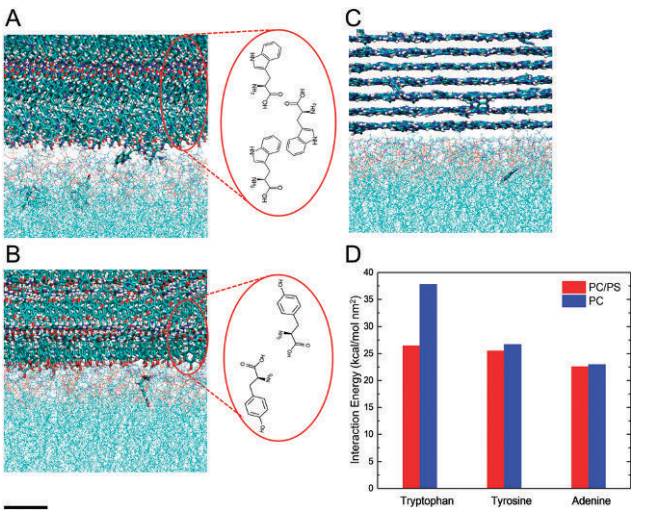


Fig. 3 Molecular dynamic simulations of the interaction of metabolite assemblies with membrane systems. (A–C) Most stable orientations for (A) tryptophan in zwitterion layer, (B) tyrosine in facet A, and (C) adenine in facet A. (D) Interaction energies of tryptophan, tyrosine, and adenine with both membranes, normalized to the contact area.

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those of facets B and C, both of which had only a limited exposure of zwitterions to the membrane. Due to the higher exposure to the polar groups when the zwitterion layer was in direct contact to the membrane, it appears that it would bind in that orientation.

Tyrosine and adenine crystal orientations relative to the membrane were also determined by hydrogen bonding and coulombic forces. In case of the tyrosine crystalline systems, the polar phenol head group bound strongly to the membranes, as seen in facet A. When the zwitterion layer was in direct contact with the PC lipid membrane, the assemblies torqued away from the orientation, as observed in the case of facets B and C. Due to facet A stability, where no significant torqueing was observed, it can be determined that in the tyrosine structures, direct contact of the phenol groups with the membranes is preferable.

In case of the adenine assemblies, due to the quadrupolar structure of the molecule, there were significantly weaker coulombic interactions, with the stronger dispersion interactions compensating. In facet A, the dispersion and coulombic interactions were of the same magnitude, unlike the other two orientations, where they were weaker. This was observed when the adenine molecules were most exposed to the membrane, attained only when the plane of the molecules was parallel to that of the membrane.

In conclusion, the experimental data here demonstrate the ability of metabolite amyloid assemblies to interact with phospholipid membranes, as previously shown in the case of their proteinaceous amyloid assemblies counterparts. Our results demonstrated the differential membrane binding ability of all three metabolite structures. We show that the tryptophan and tyrosine assemblies penetrate the membrane models, while the adenine structures bind parallel to the membrane. Similar to proteinaceous amyloids,[24–27] distinctive mechanisms underlie the membrane interaction of metabolite amyloids, while resulting in a comparable cytotoxic effect. Furthermore, molecular dynamics simulations provide a detailed atomistic information of the mechanism underlying the interaction of the metabolite assemblies with the membrane. The remarkable similarity between the mode of interaction of the metabolite amyloid assemblies and the protein ones further emphasizes the functional relationship between the two systems, supporting the definition of metabolite amyloids as an extension of the ''amyloid hypothesis''. We hope that the observations presented here will promote further exploration of the mechanism of interaction and the nature of the membrane-interacting species. As membrane interaction is assumed to play a key role in the apoptotic activity of metabolite and protein amyloids, the targeting of these interactions may be an important therapeutic direction to the future treatment of various amyloid-associated disorders.

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Conflicts of interest

There are no conflicts to declare for all authors.

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