THE METABOLITE AMYLOIDS HYPOTHESIS

Dor Zaguri[a], Shira Shaham-Niv[a], Lihi Adler-Abramovich[b], Topaz Kreiser[a], Ehud Gazit[a,c,d*]

[a]Department of Molecular Microbiology and Biotechnology
George S. Wise Faculty of Life Sciences, Tel Aviv University, Israel
dorzaguri@mail.tau.ac.il, shira.shaham@gmail.com, topazk136@gmail.com
[b]Department of Oral Biology,
The Goldschleger School of Dental Medicine, Sackler Faculty of Medicine, Tel Aviv University, Israel
LihiA@tauex.tau.ac.il
[c]Department of Materials Science and Engineering
Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Israel
[d]blavatnik Center for Drug Discovery
Tel Aviv University, Israel
ehudg@post.tau.ac.il

DOI: http://dx.medra.org/10.17374/CI.2019.6.3.23

Various metabolites which accumulate in inborn error of metabolism (IEM) disorders were shown to form amyloid-like fibrillar structures. The formed assemblies interact with model membranes, induce apoptotic cell death and are inhibited by polyphenol modulators. Finally, utilizing antibodies against the assemblies led to reduced cytotoxicity. Taken together, these findings led to the metabolite amyloids hypothesis and provides a new paradigm for IEM disorders.

L'ipotesi dei metaboliti amiliodi
Vari metaboliti che si accumulano in errori congeniti di disturbi del metabolismo (IEM) hanno dimostrato di formare strutture fibrillari simili all'amiloide. Gli assemblaggi formati interagiscono con le membrane del modello, inducono la morte cellulare apoptotica e sono inibiti dai modulatori polifenolici. Infine, l'utilizzo di anticorpi che evitino gli assemblaggi ha portato a una ridotta citotossicità. Presi insieme, questi risultati hanno condotto all'ipotesi degli amiloidi metabolici e forniscono un nuovo modello per i disturbi del IEM.

The search for the minimal self-assembly motif

Molecular self-assembly describes the spontaneous formation of ordered supramolecular structures initiated by local interactions among molecules [1]. The self-assembly process is of great significance in biology, chemistry, and material science [2]. In nature, the self-assembly processes can be observed, for example, in the formation of molecular crystals, colloids, cell

membranes and functional folded proteins [1, 2]. Polymerization of similarly folded protein subunits generates nanometer scale biological structures, such as microtubules, actin filaments and bacteria flagella. In the last two decades, the role of peptide and protein aggregation in many major human disorders was revealed [3]. Specifically, the formation of ordered amyloid fibrils has been extensively explored. It was clearly demonstrated that amyloid fibrils or their early intermediates are associated with a diverse group of diseases of unrelated etiology and pathology, including Alzheimer's disease, type II diabetes and prion disorders [4, 5]. Despite their formation by a diverse and structurally distinct group of natural proteins, as well as by synthetic proteins that also include various non-coded amino acids, all amyloid fibrils share similar biophysical and structural properties [6]. A variety of structural and biophysical studies indicate that aromatic amino acid residues are important for the acceleration of the amyloidogenic process and the stabilization of amyloid structures. Although aromatic interactions are not essential for amyloid formation, they can significantly accelerate the process, reduce the minimal association concentrations and affect the morphology of the assemblies [7-10]. It was previously shown that very short aromatic peptide fragments, as short as penta- and tetrapeptides, can form typical amyloid fibrils that share the same biophysical and structural properties of the assemblies formed by much larger polypeptides [8-11]. Additionally, the diphenylalanine peptide was shown to form various well-ordered nanostructures (Fig. 1) [12]. This short peptide represents the core recognition motif within the β-amyloid polypeptide, which forms amyloid plaques known to be associated with Alzheimer's disease. The two phenylalanine (Phe) residues (Phe19 and Phe20) in the β-amyloid peptide were suggested to mediate the intermolecular interactions between polypeptide chains. This suggestion was further substantiated by the use of Phe residues as a key component of peptide-based inhibitors of β-amyloid fibril formation [13, 14].

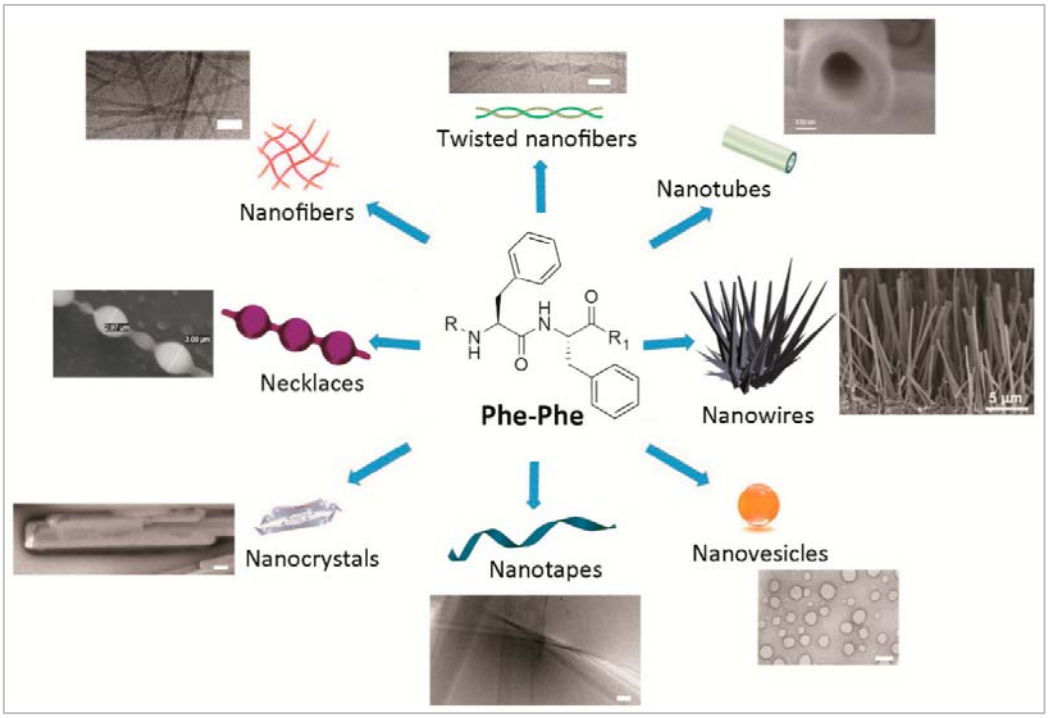

Fig. 1 - Simple compounds bearing the Phe-Phe motif self-assemble into diverse nano-morphologies. Scale bars = 100 nm, unless otherwise stated on the image. Taken from [15] by permission

Phenylalanine amyloid-like fibrils suggests a new ethology for phenylketonuria (PKU)

The formation of supramolecular entities has previously been associated with proteins and peptides. In a continuous research for a shorter assembly motif, it was demonstrated that

under pathologically-relevant millimolar concentrations, Phe, the single amino-acid can self-assemble into fibrils showing amyloid-like biophysical, biochemical and cytotoxic properties [16]. These assemblies are not irregular aggregates, as they have distinctive fibrillar morphology, visualized using electron microscopy, characteristic birefringence [17], typical ThT fluorescence emission [18, 19], and, most significantly, clear electron diffraction patterns [20, 21]. Like most amyloid structures, the assemblies formed by Phe show a dose-dependent cytotoxic effect [22-25]. This was examined by *in vitro* cellular viability experiments using a physiological range of concentrations, similar to those detected in untreated PKU individuals. At millimolar concentrations, the Phe fibrils displayed a cytotoxic effect as measured by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Moreover, when Phe fibrils were used as an antigen for several immunization cycles in rabbits, antibodies were raised against the assemblies and purified from the serum. Anti-Phe fibril antibodies were used to deplete cytotoxicity by immunoprecipitation of Phe fibrils and for immunostaining treated cells. Furthermore, histological staining in a PKU mice model, Pah-enu2, using Congo red and the Phe-fibrils specific antibody provided evidence for the presence of amyloid-like plaques in the mice hippocampus, dentate gyrus and in proximity to blood vessels. Finally, *ex-vivo,* immunohistochemistry experiments using both anti-Phe fibrils antibodies and Congo red showed indications for the presence of phenylalanine deposition in the brain tissue of PKU patients [16]. To conclude, this study paved the way for a new metabolite amyloid-like assemblies etiology, specifically for PKU and possibly in general for all inborn error of metabolism (IEM) disorders.

Inborn error of metabolism disorders and the extension of the metabolite amyloids paradigm

IEM disorders, such as PKU, are the result of cellular failure to perform critical biochemical reactions that involve numerous biological pathways. Most disorders are due to the malfunction of single genes that encode for enzymes responsible for metabolic processes [26, 27]. These abnormalities lead, in most cases, to an accumulation of one or several metabolites [28]. Six other metabolites, in addition to Phe, known to accumulate in pathological states has demonstrated their self-assembly into amyloid-like apoptosis-inducing structures [29]. Thus, adenine (Ade), cystine, orotic acid, tyrosine (Tyr), tryptophan (Trp) and uracil, all accumulating in different IEM disorders, can form ordered amyloid-like fibrils, as verified by transmission electron microscopy (TEM) and by utilizing specific amyloid dyes, such as ThT and Congo red (Fig. 2). As previously observed for protein amyloids [23, 30, 31], the metabolite assemblies demonstrated a cytotoxic effect mediated by apoptosis upon treatment of neuroblastoma cell line [29]. The apoptotic pathway was evaluated using Annexin V-FITC and PI markers and analyzed by flow cytometry. The metabolite concentration that triggers 50% of neuronal cell death was evaluated using the XTT cell proliferation assay. Importantly, no cytotoxic effect was caused by the soluble monomeric metabolites, thus confirming the role of the amyloid-like structure [29].

Metabolite amyloid-like fibrils interact with model membranes

As discussed above, metabolite amyloid assemblies possess the ability of to induce apoptotic cell death [16, 29], as previously observed for protein amyloid structures [18, 25]. Since in the case of amyloid diseases, the interaction of the amyloidogenic assemblies with the cell membrane is considered a main hallmark [33], similar mechanisms have been suggested to underlie the apoptotic effect [34]. Indeed, Phe assemblies was later demonstrated to interact with model membrane systems [35, 36].

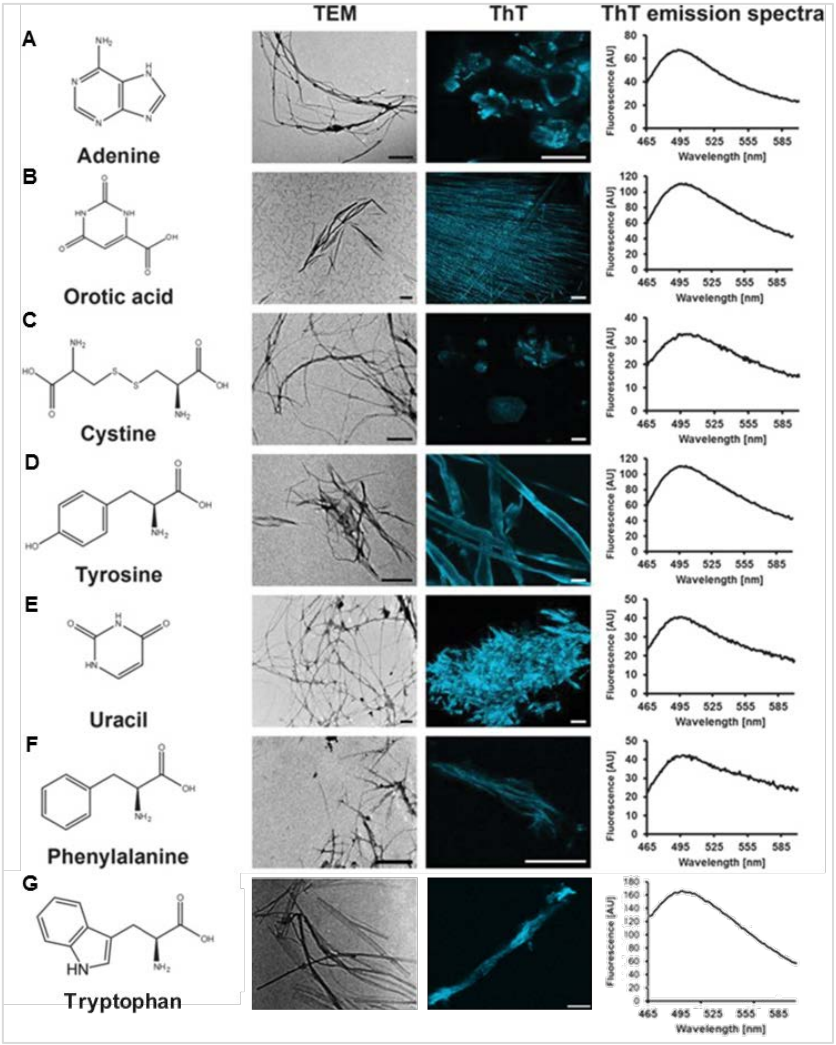

*Fig. 2 - Formation of amyloid-like structures by metabolite self-assembly. All metabolites were dissolved at 90 °C in PBS. Columns from left to right: Skeletal formula; TEM micrographs (scale bars, 500 nm); Confocal microscopy images taken immediately after the addition of the ThT reagent at a 1:1 ratio with the metabolites, with excitation and emission wavelengths of 458 and 485 nm, respectively. (Scale bars, 20 µm); ThT emission spectra (excitation at 430 nm) collected for each of the metabolites. Aged samples of each metabolite were added to 40 µM ThT in PBS to a final concentration of 20 µM ThT. (**A**) Adenine. (**B**) Orotic acid. (**C**) Cystine. (**D**) Tyrosine. (**E**) Uracil. (**F**) Phenylalanine. (G) Tryptophan. AU, arbitrary units. Modified from [29, 32] by permission*

Further examination of the interaction between metabolite amyloid assemblies and model membranes utilized a chromatic biomimetic membrane (CBM), an *in-vitro* system containing polydiacetylene (PDA) and phospholipids that accurately represent the cell membrane surfaces. CBM systems allow to the detection of membranal transformation induced by amyloidogenic assemblies via fluorescent emission shift [37, 38]. In addition, two combinations of vesicular bilayer phospholipid compositions were examined, one containing only the major component of the cell membranes (PC), and the other is known for its important role in cell cycle signalling, specifically in the case of apoptosis (PS/PC ratio). The interactions between the membrane model and Ade, Tyr and Trp fibrillar assemblies were measured by NBD [N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolaminetriethylammonium salt] label fluorescence quenching assay (Fig. 3A-F). The quenching results from bilayer penetration by membrane-reactive aggregates, whereas reduced quenching results from shielding of the NBD

dye by the vesicle-bound assemblies. Both Trp and Tyr assemblies presented strong fluorescence quenching, compared to alanine, a non-fibrillary negative control metabolite, in both lipid compositions, suggesting their ability to penetrate the bilayer membrane models. On the other hand, Ade assemblies led to a reduction in the quenching compared to the alanine control, indicating the ability of Ade fibrils to interact with the model membrane vesicles. Additionally, TMA-DPH, a sensitive probe which reacts to the dynamics of its lipid environment, was used (Fig. 3G-H) [39]. Trp assemblies induced a significant decrease in TMA-DPH fluorescence anisotropy in both phospholipid compositions, representing a higher lipid mobility and an increase in membrane fluidity. Altogether, these results imply the ability of Trp assemblies to fully penetrate the model membrane system. Tyr and Ade assemblies induced a significant decrease in TMA-DPH fluorescence anisotropy. In the case of Tyr amyloid-like assemblies, the results indicate a penetration capability, while the Ade amyloid-like assemblies appear to interact with the surface of the membrane, increasing membrane rigidity.

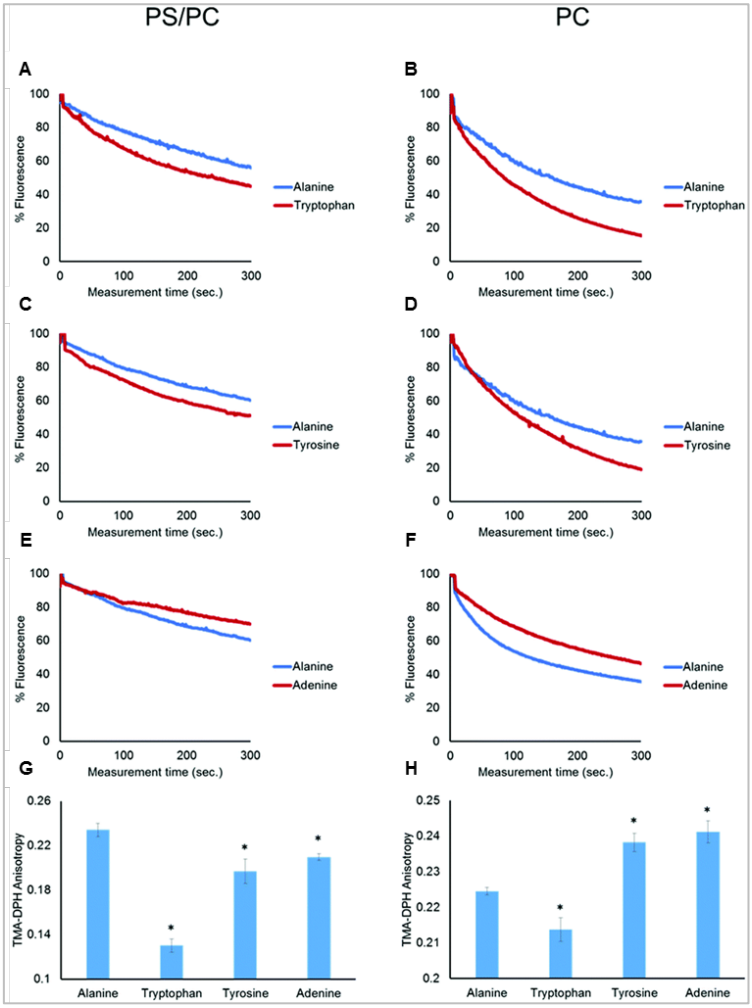

*Fig. 3 - Metabolite assemblies' interaction with membrane model systems. Tryptophan (4 mg ml^{-1}), tyrosine (4 mg ml^{-1}) and adenine (2 mg ml^{-1}) metabolite assemblies' solutions were examined. Alanine (4 mg ml^{-1}) solution was examined as a control. Two phospholipid compositions of membrane 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 as indicated, or alanine solution as a control, following the 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). Taken from [40] by permission*

Inhibition of metabolite amyloid-like fibrils formation by polyphenols

The structural similarity between protein amyloids and metabolite amyloid-like assemblies raised the intriguing possibility that the formation of both types of structures can be inhibited by the same compounds. Epigallocatechin gallate (EGCG) and tannic acid (TA) were previously shown to efficiently inhibit the formation of various protein amyloids *in vitro* and *in vivo*, and display beneficial preventive and therapeutic effects in neurodegenerative diseases [41-45]. These two polyphenolic compounds were therefore examined for their inhibition of amyloid-like fibrils formation by Phe, Tyr and Ade, the accumulation of which occurs in PKU, tyrosinemia and adenine phosphoribosyltransferase deficiency, respectively. First, the ability of EGCG and TA to effectively reduce the formation of metabolite amyloid fibrils was evaluated *in vitro* by a ThT kinetics assay. Both polyphenolic compounds showed an inhibitory effect on the different metabolite assemblies, as indicated by a significantly lower ThT fluorescence upon treatment with EGCG and TA [46]. Moreover, scanning electron microscopy (SEM) and TEM visualization of the metabolite fibrils treated with each of the polyphenol inhibitors presented a significant morphological deformation, changing from distinct supramolecular ordered structures to amorphic aggregates following the treatment. As described above, Phe, Tyr and Ade amyloid-like fibrils cause a cytotoxic effect in neuroblastoma cell lines [16, 29, 32]. On the other hand, when introduced to the same cell line, metabolite assemblies treated with EGCG or TA caused a decreased cytotoxicity and restored cell viability (Fig. 4) [46]. To deepen our understanding of the distinct inhibition mechanisms of the two inhibitors, molecular dynamics (MD) simulations were performed. The simulation of the potential inhibition activity of EGCG and TA during the formation of metabolites fibrillar assemblies calculated the interactions between inhibitor molecules and both free solvated metabolite molecules and the fibril nuclei. The computational simulations suggest that each metabolite binds to both inhibitors with similar energy.

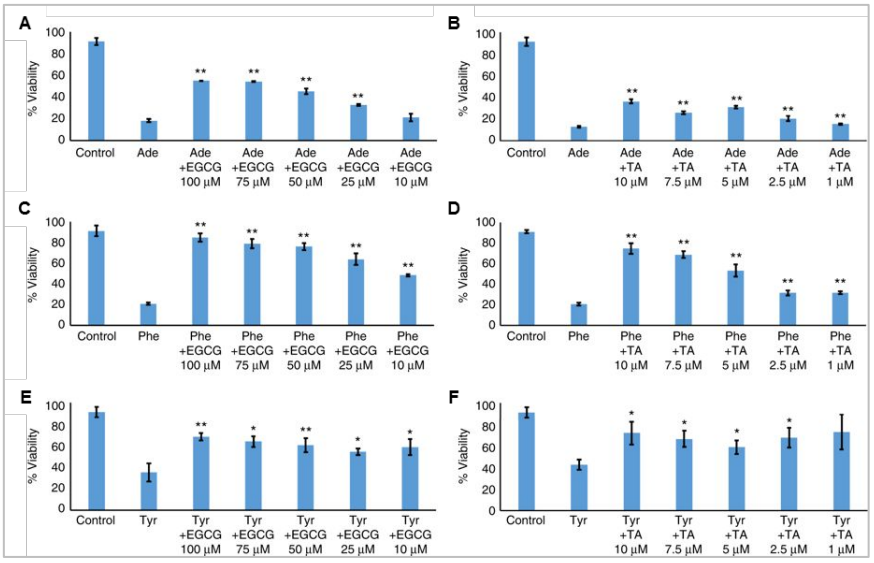

*Fig. 4 - Metabolite assemblies cytotoxicity inhibition by EGCG and TA. Ade, Phe, and Tyr were dissolved at 90 °C in DMEM/Nutrient Mixture F12 (Ham's) (1:1) without fetal bovine serum (FBS) to a final concentration of 2 mg/ml (~15 mM), 4 mg/ml (24 mM), and 2 mg/ml (10 mM), respectively, and mixed with or without the inhibitors at the stated concentrations. The control reflects medium with no metabolites, which was treated in the same manner. SH-SY5Y cells were incubated with the metabolites in the absence or presence of the inhibitors for 24 h, followed by addition of MTT reagents. Following a 4 h incubation, extraction buffer was added for an additional 0.5 h incubation, and absorbance was determined at 570 nm. The data are presented as mean ±SD. The results represent three biological repeats (*p < 0.05 **p < 0.01) (A-B) Adenine (Ade) in the presence or absence of (A) EGCG and (B) TA. (C-D) Phenylalanine (Phe) in the presence or absence of (C) EGCG and (D) TA. (E-F) Tyrosine (Tyr) in the presence or absence of € EGCG and (F) TA. Taken from [46] by permission*

The simple chemical conformation of the metabolites and the information about their molecular packing revealed the mechanism of amyloid formation [42]. The inhibition of metabolite amyloids formation by known proteinaceous amyloid inhibitors further substantiates the concept of non-protein based amyloid structures. Finally, the inhibitory properties of both polyphenols may lead to the development of novel therapeutic strategies. This will add new insights to the metabolite amyloids paradigm as it bridges between IEM and amyloid diseases.

Anti-Tyrosine fibrils antibodies reduce cytotoxicity and allow cellular imaging

Aiming to develop a novel treatment for amyloidogenic diseases by depletion of amyloid deposits, attempts have been made to harness the humoral immune response and the production of antibodies [47-50]. As described above, Anti-Phe antibodies were utilized for immunostaining, *ex vivo* immunohistochemistry, depletion of cytotoxicity and cell culture imaging. Moreover anti-Phe fibrils antibodies were shown to identify Phe assemblies in the brains of PKU patients [16]. To generate a similarly indicative tool for the three types of tyrosinemia, IEM disorders leading to the accumulation of Tyr, all resulting from autosomal recessive mutations in several genes in the Tyr metabolic pathway [51]. specific antibodies against Tyr assemblies were produced and utilized for immunodetection of Tyr structures in both *in vitro* and cell culture systems [52].

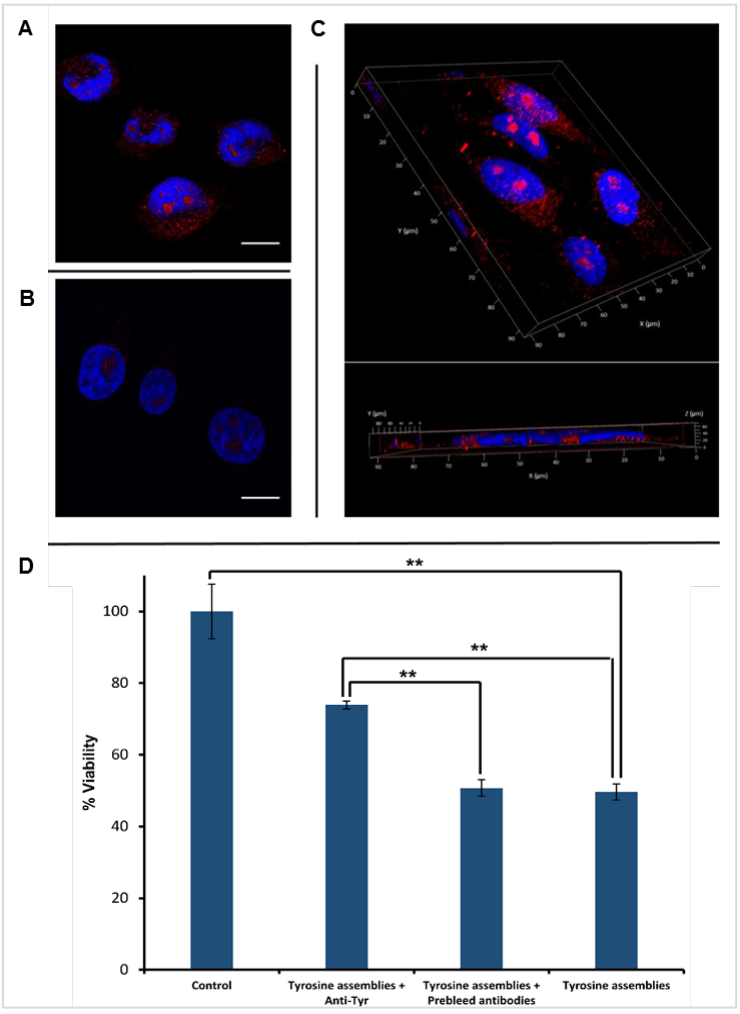

*Fig. 5 - Cellular internalization and cytotoxicity of Tyr assemblies. (A-C) Tyr was dissolved at 90 °C in cell culture medium, followed by gradual cooling of the solution. The control reflects medium with no Tyr assemblies, which was treated in the same manner. The cells were stained with anti-Tyr antibodies and visualized using confocal microscopy. DAPI (blue) and anti-Tyr staining (red) are shown. Scale bars: 15 µm. (A) SH-SY5Y cells treated with medium containing Tyr assemblies. (B) Control untreated SH-SY5Y cells. (C) 3D volume reconstruction of the Z-series with XZ-slice projection of treated cells. The interval between individual Z-stack serial images was 0.5 µm. (D) SH-SY5Y cells were treated with medium containing Tyr fibrils, which were pre-incubated with anti-Tyr antibodies (Tyrosine assemblies + anti-Tyr), Tyr fibrils pre-incubated with pre-immune antibodies (Tyrosine assemblies + pre-immune antibodies), and with medium containing non-treated Tyr fibrils (Tyrosine assemblies) for overnight incubation, followed by addition of the MTT reagent. Absorbance was determined at 570 nm and 680 nm. The results represent three biological repeats. Values are means ±SD, student's t-test, **p<0.001. Modified from [52] by permission*

Dot blot immunoassay verified the specificity of the antibodies, as anti-Tyr antibodies identified Tyr only in the assembled state. Tyr assemblies treated with the polyphenolic inhibitors EGCG and TA [46] produced a negative immune-signal, suggesting that the conformational changes caused by the inhibitors prohibited immunodetection. Furthermore, the antibodies were used for immunostaining of cultured neuroblastoma cells treated with Tyr assemblies. 3D reconstruction of the cells immunostaining implied the cellular internalization of Tyr assemblies (Fig. 5A-C), as suggested by the results of the membrane interaction study [40]. Finally, pre-incubation of the Tyr fibrils with the specific antibodies led to depletion of cytotoxicity and restored ~80% cell viability, as measured by MTT cell viability assay (Fig. 5D). Taken together, anti-Tyr antibodies provide a new tool for identification, characterization and understating of the immunological properties of Tyr amyloid-like fibrils and their role in the etiology of tyrosinemia.

REFERENCES

[1] N.B. Crane, J. Tuckerman, G.N. Nielson, "Self assembly as an additive manufacturing process: opportunities and obstacles," in SFF Symposium, 2010, 747-755.
[2] G.M. Whitesides, J.P. Mathias, C.T. Seto, *Science,* 1991, **254**(5036), 1312.
[3] F. Chiti, C.M. Dobson, *Annu. Rev. Biochem*., 2006, **75**, 333.
[4] D. Eisenberg, M. Jucker, *Cell*, 2012, **148**(6), 1188.
[5] A. Kapurniotu, *ChemBioChem*, 2012, **13**(1), 27.
[6] J. C. Rochet, P. T. Lansbury, *Current Opinion in Structural Biology,* 2000, **10**(1), 60.
[7] H. Inouye, D. Sharma, W.J. Goux, D.A. Kirschner, *Biophys. J.,* 2006, **90**(5), 1774.
[8] E. Gazit, *FASEB J.,* 2002, **16**(1), 77.
[9] O.S. Makin, L.C. Serpell, *FEBS Journal,* 2005, **272**(23), 5950.
[10] E. Gazit, *Bioinformatics*, 2002, **18**(6), 880.
[11] M. Reches, Y. Porat, E. Gazit, *J. Biol. Chem.,* 2002, **277**(38), 35475.
[12] M. Reches, E. Gazit, *Science,* 2003, **300**(5619), 625.
[13] L.O. Tjernberg *et al., J. Biol. Chem.,* 1996, **271**(15), 8545.
[14] C. Soto, E.M. Sigurdsson, *Nat. Med.,* 1998, **4**(7), 623.
[15] S. Marchesan, A.V. Vargiu, K.E. Styan, *Molecules*, 2015, **20**(11), 19775.
[16] L. Adler-Abramovich *et al., Nat. Chem. Biol.,* 2012, **8**(8), 701.
[17] P. Frid, S.V. Anisimov, N. Popovic, *Brain Research Reviews,* 2007, **53**(1), 135.
[18] S. Grudzielanek *et al., J. Mol. Biol.,* 2007, **370**(2), 372.
[19] R. Khurana *et al.*, *J. Struct. Biol.,* 2005, **151**(3), 229.
[20] L. Adler-Abramovich, E. Gazit, *Chem. Soc. Rev.,* 2014, **43**(20), 6881.
[21] A.W. P. Fitzpatrick *et al.*, *Proc. Natl. Acad. Sci.,* 2013, **110**(14), 5468.
[22] P. Salahuddin, M.T. Fatima, A.S. Abdelhameed, S. Nusrat, R.H. Khan, *European Journal of Medicinal Chemistry,* 2016, **114**, 41
[23] M. Kolarova, F. García-Sierra, A. Bartos, J. Ricny, D. Ripova, *International Journal of Alzheimer's Disease.* 2012; http://dx.doi.org/10.1155/2012/731526
[24] Y. Bram *et al.*, *Sci. Rep.,* 2014, **4**; https://www.nature.com/articles/srep04267.
[25] D.T. Loo, A. Copani, C.J. Pike, E.R. Whittemore, A.J. Walencewicz, C.W. Cotman, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, **90**(17), 7951.
[26] P.A. Levy, *Pediatr. Rev.,* 2009, **30**(4), 131; quiz 137-138.
[27] P.A. Levy, *Pediatr. Rev.,* 2009, **30**(4), e22-e28.
[28] J.M. Saudubray, G. Van Den Berghe, J.H. Walter, Inborn metabolic diseases diagnosis and treatment, Springer, 2012.
[29] S. Shaham-Niv, L. Adler-Abramovich, L. Schnaider, E. Gazit, *Sci. Adv.,* 2015, **1**(7), e1500137.
[30] N. Cremades *et al.*, *Cell*, 2012, **149**(5), 1048.
[31] S.I.A. Cohen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.,* 2013, **110**(24), 9758.
[32] S. Shaham-Niv, P. Rehak, L. Vuković, L. Adler-Abramovich, P. Král, E. Gazit, *Isr. J. Chem.,* 2017, **57**(7), 729.
[33] R. Jelinek, T. Sheynis, *Curr. Protein Pept. Sci.*, 2010, **11**(5), 372.
[34] Y. Porat, A. Abramowitz, E. Gazit, *Chemical Biology and Drug Design,* 2006, **67**(1), 27.

[35] A.S. Rosa, A.C. Cutro, M.A. Frías, E.A. Disalvo, *J. Phys. Chem. B,* 2015, **119**(52), 15844.
[36] K. Sankaranarayanan, *Soft Mater.*, 2015, **13**(4), 219.
[37] R. Jelinek, S. Kolusheva, *Biotechnology Advances*, 2001, **19**(2), 109.
[38] Z. Oren, J. Ramesh, D. Avrahami, N. Suryaprakash, Y. Shai, R. Jelinek, *Eur. J. Biochem.,* 2002, **269**(16), 3869.
[39] T. B. Woolf, B. Roux, *Proteins Struct. Funct. Genet.*, 1996, **24**(1), 92.
[40] S. Shaham-Niv, P. Rehak, D. Zaguri, S. Kolusheva, P. Král, E. Gazit, *Chem. Commun.,* 2018, **54**, 4561.
[41] F.L. Palhano, J. Lee, N P. Grimster, J.W. Kelly, *J. Am. Chem. Soc.,* 2013, **135**(20), 7503.
[42] J. Bieschke *et al.*, *Proc. Natl. Acad. Sci.,* 2010, **107**(17), 7710.
[43] K. Ono, K. Hasegawa, H. Naiki, M. Yamada, *Biochim. Biophys. Acta - Mol. Basis Dis.,* 2004, 1690(3), 193.
[44] D.E. Ehrnhoefer *et al.*, *Nat. Struct. Mol. Biol.,* 2008, **15**(6), 558.
[45] T. Mori *et al.*, *J. Biol. Chem.*, 2012, **287**(9), 6912.
[46] S. Shaham-Niv, P. Rehak, D. Zaguri, A. Levin, L. Adler-Abramovich, L. Vuković, P. Král, E. Gazit, *Commun. Chem.,* 2018, **1**(25); https://www.nature.com/articles/s42004-018-0025-z.
[47] M.D. Kazatchkine, S.V. Kaveri, *N. Engl. J. Med.,* 2001, **345**(10), 747.
[48] F. Bard *et al.*, *Nat. Med.,* 2000, **6**(8), 916.
[49] M. Stravalaci *et al.*, *J. Alzheimer's Dis.,* 2016, **53**(4), 1485.
[50] V. Geylis, M. Steinitz, *Autoimmunity Reviews,* 2006, **5**(1), 33.
[51] P.A. Russo, G.A. Mitchell, R.M. Tanguay, *Pediatric and Developmental Pathology,* 2001, **4**(3), 212.
[52] D. Zaguri, T. Kreiser, S. Shaham-Niv, E. Gazit, *Molecules*, 2018, **23**(6); https://www.ncbi.nlm.nih.gov/pubmed/29861432.