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In vitro studies of membrane permeability induced by amyloidogenic polypeptides using large unilamellar vesicles

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Summary

The process of amyloid formation is cytotoxic and contributes to a wide range of human diseases, but the mechanisms of amyloid induced cytotoxicity are not well understood. It has been proposed that amyloidogenic peptides exert their toxic effects by damaging membranes. Membrane disruption is clearly not the only mechanism of toxicity, but the literature suggests that loss of membrane integrity may be a contributing factor. In this chapter we describe the measurement of *in vitro* membrane leakage induced by amyloidogenic proteins via the use of model vesicles. We use islet amyloid polypeptide (IAPP, Amylin) as an example, but the methods are general.

Keywords

Amyloid; Membrane disruption; Cytotoxicity; Islet amyloid polypeptide; Membrane leakage

1. Introduction

"Amyloidoses" are protein misfolding diseases that are caused by the transformation of normally soluble proteins or polypeptides into partially ordered insoluble amyloid fibrils. More than thirty different proteins or polypeptides form amyloid deposits that are associated with human disorders, including neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, and metabolic diseases such as type 2 diabetes (1–4). The mechanisms of cytotoxicity are not well understood and multiple mechanisms are likely operative *in vivo*, but the literature suggests that the loss of membrane integrity may contribute to toxicity (5–8). This has motivated studies of membrane disruption by amyloidogenic proteins, work which builds on the broader literature on membrane active peptides and proteins. This chapter describes the methodology used to characterize membrane leakage induced by amyloid formation *in vitro*. Islet amyloid polypeptide (IAPP, also known as Amylin) is used as an example, but the methods are general. IAPP is a

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neuropancreatic hormone that plays a role in regulating energy metabolism. The 37-residue polypeptide is stored in the β-cell secretory granules, and is secreted with insulin (9–11). The polypeptide aggregates by an unknown mechanism in type 2 diabetes and is responsible for pancreatic islet amyloid in the disease. Amyloid formation by human IAPP (hIAPP) is toxic to islet β-cells, induces β-cell dysfunction in type 2 diabetes and plays a significant role in the failure of islet transplants (12–14). A wide range of mechanisms of hIAPP-induced cytotoxicity have been proposed, including receptor-mediated mechanisms, permeabilization of the plasma and mitochondria membranes, ER stress, defects in the unfolded protein response and defects in autophagy (4, 15).

hIAPP is a hydrophobic polypeptide and is cationic at physiological pH and, as expected, interacts with anionic membranes. Interactions of hIAPP with model membranes containing a significant portion of anionic lipids such as phosphatidylglycerol (PG) or phosphatidylserine (PS) have been widely studied. Anionic lipid vesicles, supported bilayers and monolayers accelerate amyloid formation by hIAPP, with larger effects being observed for higher percentages of anionic lipids. hIAPP promotes membrane leakage in these systems (16–19). However it is important to note that non-cytotoxic variants of IAPP can also efficiently promote leakage of model membranes, making the conduction between reductionist *in vitro* studies with simplified model membranes and the situation *in vivo* ambiguous (20). Here we describe methods employed to examine the ability of hIAPP to induce leakage of anionic model membrane systems consisting of large unilamellar vesicles (LUVs) made of mixtures of PG with the zwitterionic lipid phosphocholine (PC). The mole percent of anionic lipid typically ranges from 50 to 20 % in the most common model membrane systems used for studies of IAPP membrane interactions (16–17). In the described protocol, we use a 25 mole % anionic model membrane system as an example, but the methods are not limited to a specific composition and can be applied to other stable vesicles.

2. Materials

Deionized water and analytical grade reagents are used. Appropriate waste disposal regulations should be followed when disposing of waste materials and appropriate personal protective equipments (including goggles) should be worn and all MSDS data sheets should be carefully checked before using any reagents or solvents.

1. hIAPP is typically prepared by solid phase peptide synthesis since the peptide is toxic to many cell lines, prone to aggregate and the C-terminus is amidated. Ongoing efforts in a number of laboratories are aimed at developing improved expression systems for IAPP. The molecule can be synthesized using either (*tert*-Butyl carbamate) t-Boc or 9-fluornylmethoxycarbonyl (Fmoc) chemistry (*see* Note 1). The Alzheimer's Aβ peptide is also often prepared by solid phase peptide synthesis, although recombinant methodologies are also used. hIAPP is purified by reverse-phase HPLC using a C18 preparative column (*see* Note 2).

[1]The IAPP samples used in this example are prepared using Fmoc chemistry and Fmoc protected pseudoproline dipeptide derivatives are incorporated to facilitate the synthesis and prevent on-resin aggregation. The disulfide bond in IAPP between residues Cys-2 and Cys-7 is formed via oxidation by DMSO in the present example (23).

The identity of the pure peptide should be confirmed by mass spectrometry. IAPP and some other amyloidogenic peptides can undergo spontaneous deamidation in which Asn residues are transformed into mixtures of L-Asp, D-Asp, L-sio-Asp, D-iso-Asp (21). Thus, it is important to check the integrity of the polypeptide before commencing experiments. The peptide is best stored as a dry powder at −20 °C.

2. Lipids: For the example described here, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl sn glycerol-3phospho-(1′-rac-glycerol) (DOPG) were obtained from Avanti Polar Lipids and used without further purification. Stock solutions of lipids are prepared in chloroform and stored at −80°C (*see* Note 3 and 4).

3. Carboxyfluorescein, DMSO, Triton-X, and hexafluoroisopropanol (HFIP) were obtained from Sigma-Aldrich. Other vendors are available. Reagents should be of the highest possible grade.

3. Methods

All procedures should be carried out at room temperature unless otherwise specified.

3.1 Protein sample preparation

Handling amyloidogenic proteins can be a challenge and conflicting reports in the literature on the biophysical and cytotoxic properties of these molecules result, in part, from differences in the protocols used to solublize the protein of interest. A range of methods have been developed to prepare amyloidogenic polypeptides and proteins in initially monomeric states. Note that in many cases, low order oligomers are detected essentially as soon as the polypeptide is dissolved, thus it can be difficult to be certain that one is starting an experiment from a monomeric state. The details of the methods used for preparing the samples are specific to the protein of interest. The example described below is appropriate for IAPP and the reader is referred to the literature for protocols employed for other proteins.

1. Dried hIAPP is dissolved in 100% HFIP to prepare stock solutions and incubated for at least twelve hours. The stock solution is filtered through a 0.22 um filter (*see* Note 5).

2. Aliquots of HFIP stock solutions are freeze dried using a lyophilizer to remove organic solvents before the samples are dissolved in aqueous buffer. The strongest possible vacuum should be employed and samples should be dried at least overnight since trace amounts of residual organic solvents can influence the properties of the peptide.

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[2]HCl should be used as the ion-pairing agent instead of TFA during HPLC purification of IAPP since TFA can cause problems with cell toxicity assays and it has been shown that TFA influences the aggregation kinetics of some IAPP derived peptides (24).
[3]To prevent potential decomposition of the lipids, they should be stored in the dry form at −80 °C in case long-term storage is required.
[4]Glassware, not plastic ware, should be used when handling organic solvents such as chloroform.
[5]Filtration is required to remove any IAPP pre-fibrillar materials. This is an important step for preparing IAPP samples.

3. After overnight lyophilization, the peptide is re-dissolved in 20 mM Tris-HCl, 100 mM NaCl (pH 7.4) buffer at the desired concentration (*see* Note 6). Other buffers may be used, but the process of amyloid formation by IAPP is strongly pH dependent and is significantly faster when the side chain of His-18 and the N-terminus are neutral. The rate of hIAPP amyloid formation is also strongly dependent on ionic strength and the nature of the anion.

3.2 Preparation of LUVs

The model membrane system used in this example contains 25% anionic lipids by mole percent (*see* Note 7).

1. Chloroform stock solutions of DOPC and DOPG are transferred into a round-bottom glass flask at a 3:1 molar ratio (*see* Note 8). The organic solvent is evaporated first using a stream of nitrogen gas to form a film at the bottom of the flask (it is recommended to use the highest purity nitrogen and to employ an oil free regulator), and then further dried under a vacuum overnight in order to completely remove residual organic solvent.
2. The resulting lipid film is dissolved in 20 mM Tris-HCl and 100 mM NaCl (pH 7.4) buffer and agitated for one hour (stirring or mild shaking) (*see* Note 9).
3. After hydration, the lipid suspension is subjected to 10 freeze-thaw cycles and then extruded 15 times through 100 nm pore size filters (Whatman, GE) (*see* Note 10 and 11).
4. The phospholipid concentration of the resulting LUVs can be determined using the method of Stewart (22).
5. Fluorescent vesicles are used for the membrane disruption assays. Fluorescent LUVs incorporating the dye carboxyfluorescein are made using the same protocol described above, except that the dried lipid film is rehydrated with a buffer containing 70 mM carboxyfluorescein, 20 mM Tris-HCl and 100 mM NaCl (pH 7.4) (*see* Note 12). Carboxyfluorescein is a relatively small molecule

[6]The concentration of the peptide buffer solution should be determined to check for any loss during filtration. The concentration can be estimated by measuring the UV absorbance at 280 nm. hIAPP contains one Tyr and 2 Phe residues, but no Trp, thus the extinction coefficient at 280 nm is dominated by the absorbance of the Tyr. A precise extinction coefficient has not been reported for hIAPP, but there will be only a small uncertainty induced by using the standard value for a single Tyr. Peptide concentration can also be determined by quantitative amino acid analysis or by using the Bradford assay (25–26).

[7]The lipid composition can be altered from what described here, but the general preparation method can be used for other symmetric lipid vesicles. Here we use a 25% anionic membrane system as an example in order to describe the procedures. More complicated lipid mixtures, including ones containing cholesterol, can be prepared and methods are emerging for the preparation of asymmetric LUVs to better mimic the plasma membrane (27).

[8]The lipids must be mixed thoroughly to obtain a homogeneous solution when preparing membranes with a mixed lipid composition.

[9]There is a gel-liquid crystal transition temperature (Tc or Tm) for each lipid. For the hydration step, the lipid suspension needs to be maintained at a temperature above the highest Tc of any of the mixed lipids.

[10]Extrusion is normally used to form large unilamellar vesicles, while small unilamellar vesicles with diameters between 15 to 50 nm are usually prepared by sonication. The pore size of the filter used depends on the desired size of the lipid vesicles (typically in the range of 200 to 1000 nm for LUVs). Extrusion needs to be performed at a temperature above the Tc of the mixed lipids.

[11]LUVs can be stable for up to several days after preparation, however, it is recommended to use freshly prepared vesicles. The uniformity of the lipid vesicles can be checked by light scattering and/or by cryo electron microscopy.

[12]Carboxyfluorescein is a fluorescent dye with an excitation at 492 nm and emission of 517 nm. It is commonly used as a probe for membrane permeability. The fluorescence of the dye is self-quenched when it is encapsulated owing to the high local concentration in

and other larger probes have been developed. Comparative studies can be performed using a range of different sized probes if desired.

6. Nonencapsulated carboxyfluorescein needs to be removed from the carboxyfluorescein-filled vesicles and can be done so using size exclusion chromatography with a PD-10 column (GE Healthcare Life Sciences) and elution with 20 mM Tris-HCl 100 mM NaCl buffer (pH 7.4).

3.3 Membrane permeability measurements

A fluorescence spectrophotometer is used for the membrane leakage assay in the example presented here. A plate reader can also be used, however care must be used to avoid plates that can either disrupt model membranes or can bind IAPP.

1. The peptide solution is added to the concentrated carboxyfluorescein-filled LUVs to a final desired peptide to lipid ratio. The cuvette should be gently shaken for 3 seconds immediately after mixing. Fluorescence is measured using an excitation wavelength of 492 nm and an emission wavelength of 517 nm. A typical slit width used on the specific instrument described in this example is 1.5 nm. Time dependent studies can be performed in which the leakage is monitored by recording the carboxyfluorescein fluorescence as a function of time after addition of the peptide to the vesicles. The leakage assays should be repeated to obtain reliable estimates of the uncertainty. At least three repeats are recommended, preferably using different peptide stock solutions, to obtain mean values and apparent standard deviations.

2. For each experiment, the baseline fluorescence (Fbaseline) of the carboxyfluorescein-filled LUVs should be measured. The maximum leakage induced by total disruption of the lipid vesicles (Fmax) is determined by the addition of Triton X-100 to a final concentration of 0.2%. The percent leakage of the dye is calculated as:

$$\text{Percentage leakage} = 100 \times (\text{Ft} - \text{Fbaseline})/(\text{Fmax} - \text{Fbaseline})$$

where Ft is the measured carboxyfluorescein fluorescence.

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the interior of the vesicle. Permeabilization of the membrane allows the dye to escape, thereby lowering the concentration and relieving self-quenching. The pH of the buffer may be decreased after dissolving the compound, thus one should be sure to readjust the buffer pH to 7.4 if needed.

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