Here, we describe a robust protocol using mouse models to screen potential insulin-stabilizers and insulin moieties. We have generated a mouse model of amyloidoma, found in diabetic patients undergoing insulin therapy. This model can be used to screen potential insulin stabilizers and insulin moieties to prevent amyloidoma formation. This protocol can further be used for the preclinical validation of therapeutically relevant insulin stabilizers and formulations. The protocol highlights all the critical steps for generating amyloidoma in a preclinical model.
Protocol

Subcutaneous amyloidoma models for screening potential anti-fibrillating agents in vivo

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SUMMARY

Here, we describe a robust protocol using mouse models to screen potential insulin-stabilizers and insulin moieties. We have generated a mouse model of amyloidoma, found in diabetic patients undergoing insulin therapy. This model can be used to screen potential insulin stabilizers and insulin moieties to prevent amyloidoma formation. This protocol can further be used for the preclinical validation of therapeutically relevant insulin stabilizers and formulations. The protocol highlights all the critical steps for generating amyloidoma in a preclinical model.

For complete details on the use and execution of this profile, please refer to Mukherjee et al. (2021).

BEFORE YOU BEGIN

Diabetic patients under insulin therapy often develop insulin-derived amyloidoma at the site of repeated injections (D’Souza et al., 2014; Mayhew et al., 2017; Nagase et al., 2009; Nilsson, 2016) because commercially available injectable insulin formulations (especially fast-acting analogs) with phenolic preservatives are susceptible to heat-induced fibrillation (Ahmad et al., 2003; Carter and Heinemann, 2018; Delbeck and Heise, 2021; Woods et al., 2012). This iatrogenic amyloidosis with associated subcutaneous insulin resistance (Nagase et al., 2014; Nakamura et al., 2019) fuelled biomedical research towards developing anti-amyloidogenic insulin formulations. Subcutaneous injection of heat-induced fibrillated human insulin in mice presents an animal model to generate local amyloidoma and its subsequent study in vitro (Chinisaz et al., 2014). Here, we use IS1, a non-toxic, plasma membrane-impermeable tetrapeptide that potently protracted insulin fibrillation and amyloidoma formation in a preclinical setting (Mukherjee et al., 2021).

All animal experiments were performed according to the guidelines set by the approved Institutional Animal Ethics Committee (Approved by CPCSEA, India). 6–8 weeks old wild-type male BALB/c mice housed in a 12-h light/dark cycle at 22 ± 1°C with ad libitum access to food and water are used in these experiments. Before beginning the experiment, the body hair on the dorsal region of mice is removed to facilitate the subcutaneous injection of insulin. Insulin fibril and insulin-IS1 complex are prepared.

Preparation of insulin solution

© Timing: 10 min
1. Weigh bovine recombinant insulin in a microcentrifuge tube.

   **Note:** Human recombinant insulin as well as other commercial human insulin analogs can also be used for this study.

2. Add 15–20 μL of 50 mM Citrate phosphate buffer (pH-2.6) to the tube and mix thoroughly until it completely dissolves and the solution turns transparent.

3. Dilute it with Phosphate Buffer Saline (pH -7.4) to a final stock concentration of 350 μM.

4. Further dilute the stock solution in PBS to a working concentration of 0.875 μM.

**Preparation of insulin fibrils**

@ Timing: 4–5 h

5. Incubate the insulin solution at 62°C hot water bath in a sealed microcentrifuge tube.

6. Put the microcentrifuge tube in a shaking (100 rpm) hot water bath at 62°C for 4–5 h. Alternatively, every 15 min, the microcentrifuge tube was turned upside down 3–5 times to facilitate gentle agitation and put back at 62°C incubation for up to 5 h.

7. The solution will gradually turn turbid indicating the formation of insulin fibrils.

**Preparation of insulin: IS1 complex fibrils**

@ Timing: 4–5 h

8. Add an equimolar volume of IS1 to the working insulin solution.

9. Incubate at 62°C hot water bath in a sealed microcentrifuge tube.

10. Every 15 min, the microcentrifuge tube was turned upside down 3–5 times and again incubated at 62°C for up to 5 h.

The solution does not turn turbid as observed in the case of insulin fibrils without IS1.

**Note:** Insulin (fibrillated) and insulin with IS1(non-fibrillated) can be stored at 4°C.

**Characterization of prepared insulin fibrils and insulin-IS1 complex**

@ Timing: 5–7 h

11. Dissolve ThT in distilled water to a working solution of 2mM while the working concentration should be 20μM.

12. Incubate stock solution of insulin and insulin: IS1 complex with ThT at 62°C followed by fluorescence measurement using a microplate reader (excitation at 440 nm and emission at 480 nm). The time kinetics of insulin fibrillation were further studied for 5 h (**Figure 1**).

13. For further validation, samples concerning insulin fibrils and insulin-IS1 complex were subjected to Atomic Force Microscopy (AFM) for the visualization of the insulin fibrils/oligomers (**Figure 1**, inset).

For a detailed protocol of ThT assay and AFM studies, please consider the protocol described in the previously published paper (**Mukherjee et al., 2021**).

**Shaving mice body hair and marking the spot for insulin administration**

@ Timing: 10 min
14. Apply hair removal cream on the dorsal region of mice and set it aside for 5 min.

*Note:* Do not leave the cream on the mice’s hair for a long duration as it can cause irritations and rashes on the skin.

15. Then, with a spatula gently scrape off the hair.

16. Rinse any remnant cream with a tissue soaked in lukewarm water followed by soaking with dry wipes (Figure 2).

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**Figure 1. Characterization of prepared insulin fibrils**

ThT assay represents the fibrillation kinetics for the insulin in the presence of the increasing concentration of IS1. Inset: AFM images of heat-induced insulin fibrillation in the presence and absence of IS1; scale bar 1 μm.

Data adapted from Mukherjee et al. (2021).

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**Figure 2. Dorsal view of BALB/c mice**

(A) Unshaved.
(B) Shaved.

Scale bar 1 cm.
Calculation of dose of insulin needed to be administered

© Timing: 5 min

17. The dose of insulin to be administered to a mouse is 0.5 U/Kg of body weight (Kooptiwut et al., 2002; Morley et al., 2015; Zheng et al., 2014).

18. Weigh mice and calculate the amount of insulin to be administered to each mouse. (Refer to Table 1).

Table 1. Mice weight and calculation of amount and volume of insulin to be injected

| Mice | Weight of mice (g) | Amount of insulin to be administered (0.5 U/kg body weight) | Volume of insulin to be injected (μL) |
|------|-------------------|-------------------------------------------------------------|--------------------------------------|
| 1    | 24                | 0.012                                                       | 83.28                                |
| 2    | 34                | 0.017                                                       | 117.98                               |
| 3    | 30                | 0.015                                                       | 104.1                                |

19. The dose of insulin to be injected is calculated using the formula:

\[ \text{Amount of insulin} = \frac{\text{Weight of mouse} \times 0.5 \text{ U/Kg}}{1000} \]

20. Weigh each mouse and calculate the amount of insulin to be injected using the formula above. (Refer to Table 1).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** |
| Citric Acid         | Qualigens | Q22595     |
| Sodium Phosphate Dibasic Dihydrate | Sisco Research Laboratories | 32080     |
| Phosphate Buffer Saline | Sigma-Aldrich | P4417     |
| Sodium Chloride     | Sigma-Aldrich | S5886     |
| Sodium Hydroxide pellets purified | Merck | 106469     |
| Thioflavin T        | Sigma-Aldrich | T3516     |
| MUSCOVITE MICA –V1 | Electron Microscopy Sciences | 180309    |
| Congo Red           | Sigma-Aldrich | C6767     |
| Formaldehyde solution 37–41% w/v GR | Merck | 61783705001730 |
| Xylene              | Merck | S34056     |
| Hematoxylin QS      | Vector Laboratories | H-3404    |
| DPX New Mounting Media | Merck | 100579     |
| Ethanol             | Merck | 1.00983    |
| D-Glucose           | Qualigens | 15405     |
| Insulin from Bovine Pancreas | Sigma-Aldrich | IS5000  |
| **Experimental models: Organisms/strains** |
| Mouse strain: BALB/c | Animal House Facility, CSIR-Indian Institute of Chemical Biology | N/A       |
| **Other** |
| Insulin Syringe     | Becton Dickinson(BD) | 31-G       |
| Water Bath          | Grant | LSB 12     |
| Dry heat bath       | IKA | RCT B 5022 |
| Microtome           | N/A | N/A        |
| Mouse dissection tray | N/A | N/A        |
| Veet hair removal cream normal skin | Veet | 8901396354109 |
| Sd Codefree Blood Glucose Monitoring System,SD Biosensor | N/A |
| 100 Strips          | SD Biosensor | 01GM11    |
| Atomic Force Microscopy (AFM) | Keysight | N/A      |
| Polarized light microscope | Olympus | SXZ16     |
MATERIALS AND EQUIPMENT

Citrate Phosphate Buffer (50 mL)

| Reagent                          | Final concentration | Stock concentration | Volume (mL) |
|----------------------------------|---------------------|----------------------|-------------|
| Citric Acid                      | 44.6 mM             | 0.1 M                | 22.3        |
| Sodium Phosphate Dibasic Dihydrate| 10 mM               | 0.2 M                | 2.7         |
| Double Distilled water           | –                   | –                    | 25          |
| Total                            | –                   | –                    | 50          |

Adjust pH to 2.6 with concentrated HCl.

Note: Freshly prepared buffer should be used for the experiment.

Note: The citrate phosphate buffer can be stored at 4°C and should be used within a month.

10% Formalin

| Reagent                                      | Final concentration | Volume (mL) |
|----------------------------------------------|---------------------|-------------|
| Formaldehyde solution 37–41% w/v GR          | 10%                 | 5           |
| Phosphate Buffer Saline                      | –                   | 45          |
| Total                                        | –                   | 50          |

Note: Buffer should be freshly prepared before experiment.

Note: 10% Formalin can be stored at 15°C–30°C and to be used within three months of preparation. PBS can be stored at 15°C–30°C for 24 months.

Congo Red (100 mL)

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Congo Red | 0.5%               | 0.5 g  |
| Alcohol  | –                   | 100 mL |
| Total    | –                   | 100 mL |

Note: Congo Red solution must be freshly prepared before the experiment.

Note: The solution can be stored at room temperature (22°C–25°C) and can be stored until 6 months.

Sodium Hydroxide

| Reagent      | Final concentration | Amount |
|--------------|---------------------|--------|
| Sodium Hydroxide | 1%                 | 1 g    |
| Distilled water  | –                   | 100 mL |
| Total         | –                   | 100 mL |

Note: The sodium hydroxide solution should be freshly prepared before the experiment.

Note: The sodium hydroxide solution should be stored at 25°C in plastic containers and can be used within 30 days from preparation.
Note: Do not store the alkaline alcohol solution. It must be prepared before the experiment at room temperature and used immediately.

## STEP-BY-STEP METHOD DETAILS

### Subcutaneous administration of insulin and amyloidoma formation

© Timing: 10 min, 28 days

Here, insulin fibril is injected subcutaneously to generate amyloidoma.

This section is shown in Methods video S1.

1. Locate a spot and insert the needle subcutaneously.
2. Gently and slowly inject insulin (fibrillated) and insulin with IS1 (non-fibrillated) on the contralateral sides of the vertebrae. The area where insulin is administered will bloat which will gradually dissipate with time.
3. Repeat the injection once daily for 28 consecutive days at the same injection site until the visual appearance of skin amyloidoma (Figure 3).

Note: Insulin is readily soluble in acidic pH. Hence, insulin is first dissolved in a low volume of citrate phosphate buffer (pH-2.6).

Note: The pH of the subcutaneous region is around 7.4. Therefore, before administration of insulin in mice, it is to be diluted to the required amount in PBS (pH-7.4) since the

| Alkaline alcohol solution (100 mL) | Reagent       | Final concentration | Stock concentration | Volume (mL) |
|-----------------------------------|---------------|---------------------|---------------------|-------------|
| Sodium Hydroxide                  | 0.01%         | 1%                  | 1                   |
| Alcohol                           | 50%           | 50%                 | 99                  |
| Total                             | –             | –                   | 100                 |

Figure 3. Visualization of subcutaneous amyloid mass deposition after injections for twenty-eight consecutive days
(A) The emergence of subcutaneous amyloid mass (black arrow) at the site of insulin fibril administration, while no substantial amyloid mass was visible (red arrow) at the site of Insulin-IS1 complex administration.
(B) Zoomed view of mice exhibiting amyloid mass for insulin fibril (black arrow) and insulin-IS1 complex (red arrow). Scale bar 1 cm.
administration of acidic insulin in mice can cause lesions on mice skin since citrate is a strong ion, resulting in acidosis in the administered subcutaneous area. (troubleshooting 1)

Note: The insulin needs to be administered subcutaneously at the same site for better amyloid formation (troubleshooting 2).

△ CRITICAL: After the administration of insulin, monitor the mice to check whether it has undergone a hypoglycemic shock. To check, prick the mouse tail with a needle to obtain blood and check the blood glucose level using a glucometer (troubleshooting 3). In case of hypoglycemia, inject 100 µL of 20% glucose intraperitoneally and check whether the blood glucose level has recovered to a normoglycemic state, else, inject more glucose.

Excision of amyloidoma

⊙ Timing: 15 min

This section is shown in Methods video S2.

4. Sacrifice the mice by cervical dislocation.
5. Using scissors, carefully excise the amyloidoma along with the surrounding tissue (Figure 4).
6. Weigh the mass of the skin biopsy and note it down (Table 2).
7. Store the tissue in 10% formalin.

△ CRITICAL: Be careful to locate the position of the amyloidoma and cut the skin surrounding the amyloidoma along with it.

Fixing and sectioning tissue

⊙ Timing: 1 day

Figure 4. Excision of skin biopsy at the site of insulin administration
(A) Gross image showing mice with excised skin biopsies; Scale bar 1cm.
(B) The cross-sectional surface of the insulin fibril-induced amyloid mass, top and bottom black arrows indicating the edges of the amyloid mass (top). No amyloid formation (bottom) in Insulin-IS1 Complex.
Scale bar 3.3 mm.
Here, fixed skin biopsies are embedded in paraffin, cut into tissue sections and mounted on slides.

8. Dehydrate the tissue by immersing the tissues at room temperature (20°C–25°C) according to Table 3:
9. Carefully immerse the tissue in paraffin at 58°C.
10. Using a rotary microtome, cut tissues 10μm thick.

**Note:** The tissues should be around 10 μm thick for optimal Congo Red staining (troubleshooting 4).

11. Float the sections in a 56°C water bath.
12. Mount the sections onto slides and dry them overnight at room temperature.

**Congo red staining**

Here, amyloid fibrils are detected using Congo red dye. Congo red is a metachromatic stain that is commonly used for the detection of amyloids. This dye forms a hydrogen bridge bond with the carbohydrate part of the substrate and is capable of depositing itself on amyloid fibrils. The tissue appears orange-red upon Congo Red staining under a light microscope; however, under polarized light amyloid mass is observed as apple-green birefringence against a dark background.

**Deparaffinizing the tissue section**

© Timing: 50 min

13. Dry heat the slides at 85°C for 15–20 min to dissolve wax.
14. Deparaffinize the slides by immersing them into Coplin jars containing xylene for 2 min. Repeat the process two more times at room temperature.
15. Rehydrate the slides by serially immersing them in a gradient of alcohol at room temperature according to Table 4.
16. Wash the slides by dipping them in tap water for about 2 min.

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### Table 2. Weight of skin biopsy

| Mice     | Weight of skin biopsy where insulin fibrils is administered (mg) | Weight of skin biopsy where insulin-IS1 complex is administered (mg) |
|----------|---------------------------------------------------------------|---------------------------------------------------------------------|
| Mice 1   | 390.4                                                         | 206.1                                                               |
| Mice 2   | 480.3                                                         | 234.5                                                               |
| Mice 3   | 523.5                                                         | 224.8                                                               |

### Table 3. Reagents and corresponding incubation time for dehydration of tissues

| Reagent | Time (min) |
|---------|------------|
| 70% Ethanol | 30         |
| 70% Ethanol | 30         |
| 70% Ethanol | 30         |
| 90% Ethanol | 30         |
| 90% Ethanol | 30         |
| 100% Ethanol | 30        |
| 100% Ethanol | 30        |
| 100% Ethanol | 30        |
| Xylene   | 20         |
| Xylene   | 20         |
| Xylene   | 20         |
Note: The tissue needs to be properly deparaffinized for optimal staining. After the deparaffinization step, check whether the wax has been completely removed (troubleshooting 5).

Staining

Timing: 50 min

17. Immerse the slides in a Coplin jar containing Congo Red Solution and incubate for 15–20 min at room temperature.
18. Rinse the slides in distilled water following which quickly differentiate by dipping the slides in alkaline alcohol solution (5–10 dips).

Note: Alkaline alcohol solution facilitates selective staining of amyloid and increases the intensity of staining.

19. Rinse the slides in tap water.
20. Dehydrate the slides by immersing them in a Coplin jar containing 90% alcohol for 3 min followed by two changes of 100% alcohol for 3 min each at room temperature.
21. Clear the slides by immersing them in 2 changes of xylene for 3 min each at room temperature.
22. Mount the slides with DPX.

Polarized light microscopy

Timing: 5 min

23. Observe the slides with a polarized light microscope.

EXPECTED OUTCOMES

Daily injection of insulin fibril at the same site for 28 consecutive days led to the formation of a visible subcutaneous mass. However, insulin with IS1(non-fibrillated) administration resulted in no such visible amyloidoma. To visualize the subcutaneous fibrillar deposition, Congo Red staining is performed on the skin tissue sections excised from the site of injection. The amyloid exhibits apple-green birefringence when viewed in a polarized light microscope (Figure 5).

This protocol can be used for screening novel insulin stabilizers as well as studies involving the generation of drugs for ablating amyloidosis. In place of IS1, one can use other insulin-stabilizers or drugs that harbour the potential to ablate amyloidosis. Congo Red staining can be used to characterize the deposition of amyloids. This protocol can even be used for other pro-amyloidogenic proteins in combination with their potential modulators.

LIMITATIONS

This present study has few potential limitations. Pharmacokinetic profile of insulin stabilizer, a crucial component for future translation cannot not be discerned from the present protocol.
TROUBLESHOOTING

Problem 1
Lesions in mice skin after insulin administration. Insulin is soluble in low pH. However, subcutaneous administration of insulin solution at low pH can cause lesions on mice’s skin (step 2 in step-by-step method details).

Potential solution
After dissolving the insulin with low volume of acidic solvent (e.g., Citrate Phosphate Buffer (pH-2.6)), dilute it in PBS (pH-7.4).

Problem 2
Amyloid mass not formed. This may occur if the injection is not administered at the same site. If insulin fibrils are injected at different sites, the fibril instead of accumulating will gradually dissipate thus hampering amyloidoma formation (step 3 in step-by-step method details).

Potential solution
For convenience, mark the site of injection and try to inject at the same spot every day. This will lead to the amyloid mass formation.

Problem 3
Hypoglycemic shock in mice. In some cases, bioactive insulin administration can lead to hypoglycemia in mice (step 2 and 3 in step-by-step method details).
Potential solution
In case of acute hypoglycemic shock, rapid intraperitoneal administration of 100 μL 20% glucose solution followed by blood glucose monitoring is essential for the recovery of mice to a normoglycemic condition.

Problem 4
The thickness of the tissue section. If the tissue section is too thin, otherwise, it can hinder the optimal staining (step 10 in step-by-step method details).

Potential solution
The tissue section should be around 10 μm thick for optimal staining.

Problem 5
Deparaffinization of tissue sections. Tissue sections are not completely dewaxed and hence optimal Congo Red staining will not be obtained (step 13–15 in step-by-step method details).

Potential solution
The incubation time for the deparaffinized steps can be increased and the slides should be checked for proper dewaxing.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Partha Chakrabarti (pchakrabarti@iicb.res.in).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any datasets or code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101027.

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AUTHOR CONTRIBUTIONS
D.D., A.P., and S.K.M., performed the experiments. S.C. and P.C. conceived and designed the protocol. D.D. and P.C. wrote the protocol.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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