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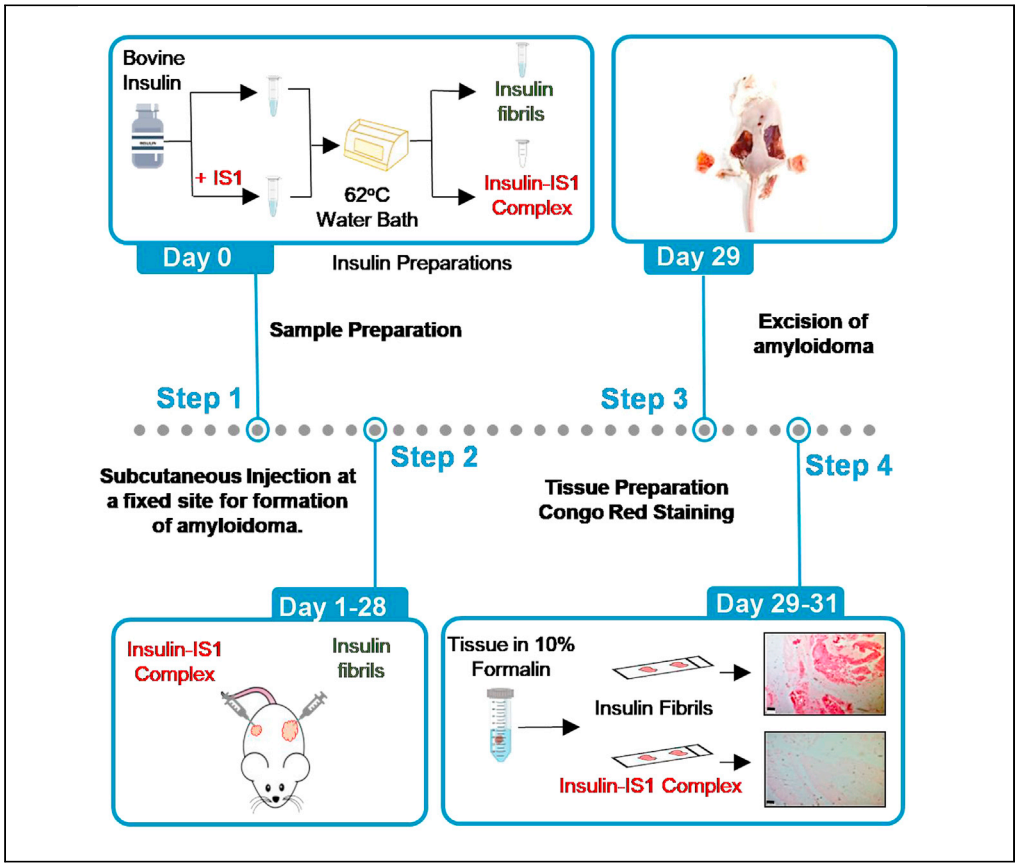
Subcutaneous amyloidoma models for screening potential anti-fibrillating agents in vivo



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.

Debajyoti Das, Avishek Paul, Sujay Krishna Maity, Subhrangsu Chatterjee, Partha Chakrabarti

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Highlights

We present a detailed protocol for the generation of subcutaneous amyloidoma in mice

This protocol facilitates screening of novel insulin-stabilizing molecules *in vivo*

This approach can be adapted to study amyloidosis in other amyloid-related diseases

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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

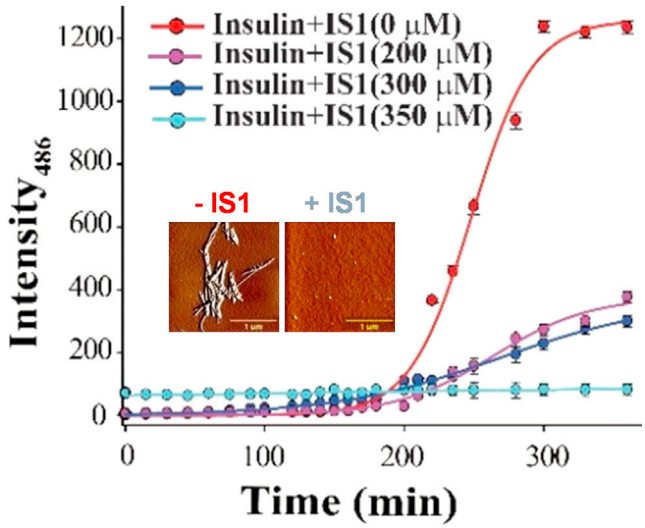

Figure 1. Characterization of prepared insulin fibrils

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).

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).

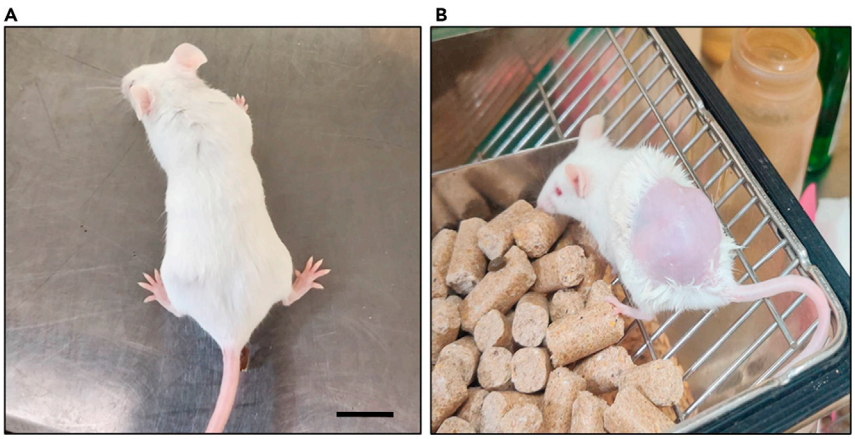

Figure 2. Dorsal view of BALB/c mice

(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)
Mice 1	24	0.012	83.28
Mice 2	34	0.017	117.98
Mice 3	30	0.015	104.1

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	534056
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	I5500
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 S022
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, 100 Strips	SD Biosensor	N/A
SD CodeFree Blood Glucosemeter	SD Biosensor	01GM11
Atomic Force Microscopy (AFM)	Keysight	N/A
Polarized light microscope	Olympus	SZX16

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.

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

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

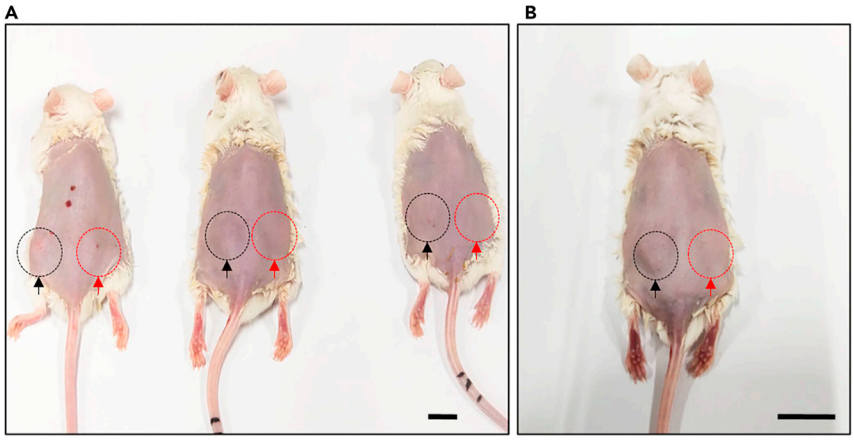

Figure 3. Visualization of subcutaneous amyloid mass deposition after injections for twenty-eight consecutive days
(A) The emergence of subcutaneous amyloid mass (black arrow) at the site of insulin fibril administration, while no substantial amyloid mass was visible (red arrow) at the site of Insulin-IS1 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.

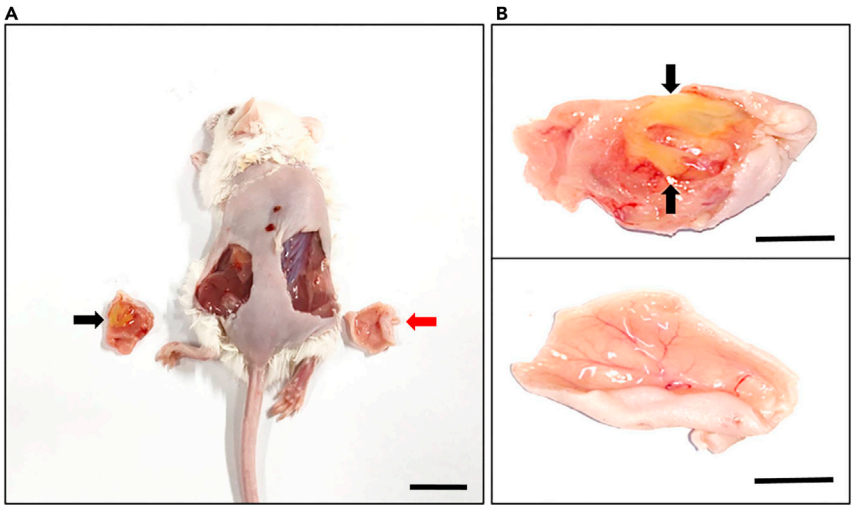

Figure 4. Excision of skin biopsy at the site of insulin administration
(A) Gross image showing mice with excised skin biopsies; Scale bar 1cm.
(B) The cross-sectional surface of the insulin fibril-induced amyloid mass, top and bottom black arrows indicating the edges of the amyloid mass (top). No amyloid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

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

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

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 (trouble-shooting 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.

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

Table 4. Reagents and corresponding incubation time for rehydration of tissue section

Reagents	100% Ethanol	100% Ethanol	95% Ethanol	85% Ethanol	75% Ethanol	50% Ethanol
Time (min)	7	5	5	5	5	5

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.

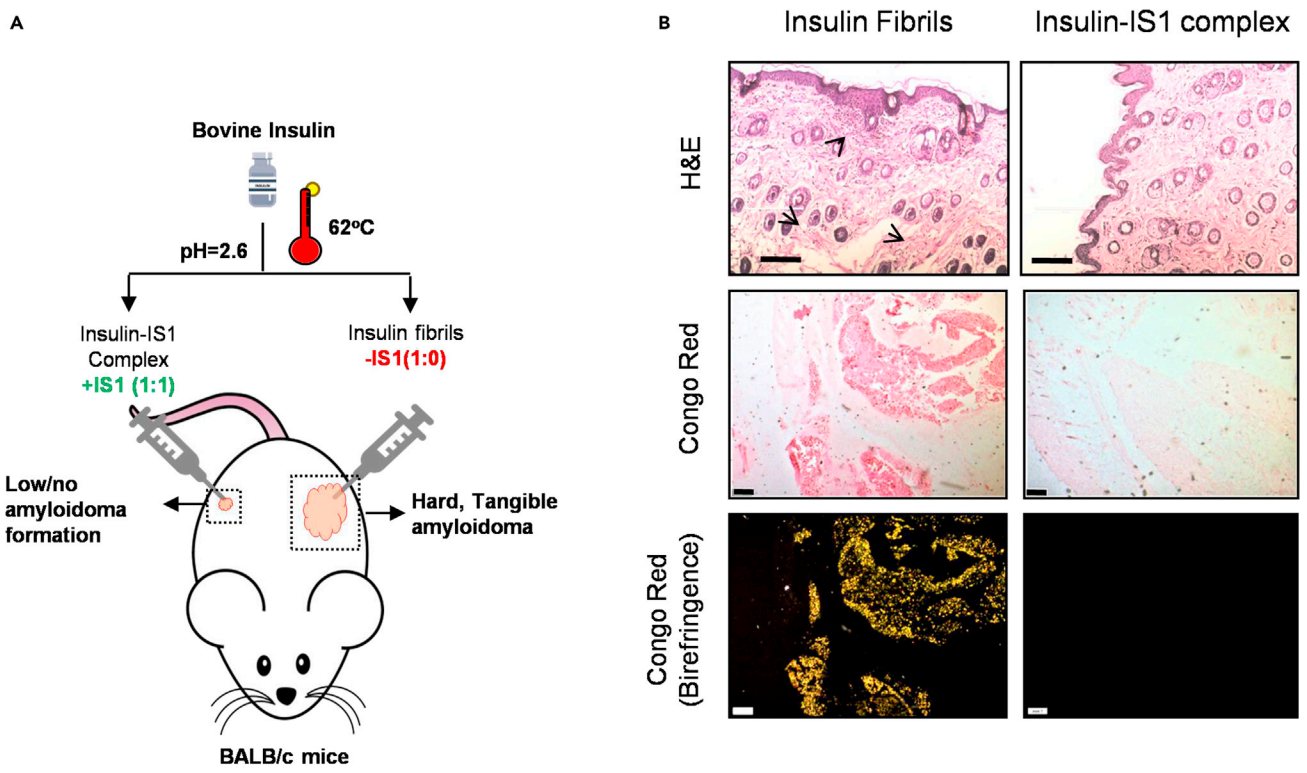

Figure 5. Workflow of subcutaneous amyloidoma formation and histological characterization

(A) Schematics representing the workflow involved in screening insulin-stabilizers by amyloidoma formation in BALB/c mice.

(B) Skin biopsies stained with hematoxylin and eosin (H&E) and Congo Red visualized using light and polarized light microscopy exhibits Amyloid deposition for insulin fibrils (left panel) and Insulin-IS1 complex (right panel).

Scale bar 1 mm.

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.

ACKNOWLEDGMENTS

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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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