

Preparation and Evaluation of Alpha-2-Macroglobulin Hydrogel for Drug Release Kinetics and Biocompatibility on Human Gingival Fibroblasts: An In-vitro Study

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ABSTRACT

Introduction: Periodontitis is a chronic inflammatory disease characterised by the destruction of the supportive tissues of the teeth. Conventional treatments often involve systemic or topical drug administration, which can result in non-specific drug delivery. The use of localised drug delivery systems, such as hydrogels, offers a promising solution for targeted treatment, improving therapeutic outcomes and minimising side effects.

Aim: This study investigates the preparation, drug release pattern, and biocompatibility of Alpha-2-Macroglobulin (A2M) hydrogel for use as a Local Drug Delivery (LDD) agent in the management of periodontitis.

Materials and Methods: This in-vitro study was conducted at Department of Periodontics, Saveetha Dental College, Saveetha Institute of Technical and Medical Sciences (SIMATS), Chennai, Tamil Nadu, India, between April 2024 and August 2024. A2M hydrogel preparation and in-vitro assays were carried out in a laboratory setup over a one-month period. The A2M hydrogel was prepared using Pluronic F127 and chitosan polymer. The biocompatibility of the hydrogel was evaluated through cytotoxicity assays on Human Gingival Fibroblasts (HGFs). The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay was used to evaluate cell viability, and the drug release pattern was analysed. For MTT analysis at various time intervals, three groups were compared: A2M hydrogel, pure hydrogel (without A2M), and a control group (sterile water). Data were analysed using the Statistical Package for the Social Sciences (SPSS, Version 23.0). Intergroup comparisons were performed using one-way ANOVA, followed by Tukey's post hoc test for pairwise analysis. A p-value of ≤ 0.05 was considered statistically significant.

Results: The A2M hydrogel showed an initial burst release in the first few hours, followed by controlled and sustained release in the subsequent hours. Cytotoxicity tests revealed that the hydrogel was non toxic at concentrations up to 400 micrograms/mL, with cell viability rates exceeding 85%. Additionally, the hydrogel supported cell adhesion and proliferation, suggesting its suitability for periodontal regeneration.

Conclusion: A2M hydrogel exhibits promising characteristics as a localised drug delivery system, shown by its capacity to deliver medications under controlled conditions. Its biocompatibility makes it an attractive candidate for further exploration in clinical applications.

Keywords: Host modulation therapy, Periodontal disease, Protease inhibitors

INTRODUCTION

Periodontitis is a multifactorial chronic inflammatory disease that damages the supportive tissues of the teeth, including the periodontal ligament, alveolar bone, and cementum. Although periodontitis is primarily caused by the accumulation of bacteria and their by-products, the host response to periodontopathogens plays an important role in disease progression, leading to tissue destruction. If left untreated, this condition can result in tooth loss [1]. The presence of microbes triggers the host immune system, leading to the release of various proteases, especially metalloproteinases. This causes the degradation of extracellular matrix components such as collagen, resulting in the breakdown of periodontal ligament and gingival tissues [2].

Homeostatic mechanisms regulate the level of these proteases by releasing protease inhibitors, for example, Tissue Inhibitor Metalloproteinase (TIMP), A2M [3]. Host modulation aims to modify the host's immune response, reducing inflammation, slowing disease progression, and enhancing tissue healing. High molecular weight plasma protease inhibitor A2M is thought to be crucial for defense systems that try to stop and eliminate endoproteases that are damaging due to proteolysis, whether they are endogenous or exogenous [4]. Because A2M is found in bodily fluids and helps regulate the proteolytic activity of the extravascular area, its protective

function is not limited to the blood circulation. Because it interacts with a wide range of proteinases and has the ability to absorb some proteases attached to α_1 -antitrypsin, A2M is significant among protease inhibitors [5]. Though activity of proteases are regulated by protease inhibitors, a loss of balance where proteases outnumber protease inhibitors leads to excessive tissue breakdown.

Despite advancements in periodontal treatments, including mechanical debridement and systemic antibiotics, the effective and localised delivery of therapeutic agents remains a challenge. Issues such as poor bioavailability, systemic side effects, and the inability to achieve sustained drug release at the site of disease have gained attention as promising alternatives, providing advantages such as targeted therapy, reduced side effects, and sustained release of therapeutic agents directly at the site of infection [6]. Hydrogels, which are networks of three dimensional polymers that can retain significant amounts of water, are particularly attractive for LDD due to their biocompatibility, ease of application, and ability to deliver drugs over extended periods [7].

Among the various options for hydrogel development, A2M, a natural plasma protein protease inhibitor, has garnered interest because of its anti-inflammatory properties, ability to bind and neutralise proteases, and its role in tissue regeneration [8]. Moreover, it modulates the host response by regulating a wide range of

proteases, particularly metalloproteinases present in periodontal pockets, and inhibits excessive tissue destruction. A2M's potential for enhancing wound healing and reducing tissue damage in periodontal diseases makes it an ideal candidate for developing a novel hydrogel formulation for periodontitis therapy.

The preparation of an A2M based hydrogel allows for the formation of a crosslinked network that enables controlled drug release, boosting the effectiveness of active ingredients such as growth factors, antibiotics, or anti-inflammatory medications [9-11]. The biocompatibility of these hydrogels is a critical factor for their clinical application, as they must not induce adverse immune responses while promoting tissue repair and regeneration. The controlled release properties are influenced by several factors, including the hydrogel's polymer composition, crosslinking density, and the physicochemical interactions between the drug and the hydrogel matrix [9,12].

In this study, we focus on the preparation process of A2M hydrogel, the biocompatibility of the hydrogel, and its drug release pattern.

MATERIALS AND METHODS

The in-vitro study was conducted at Department of Periodontics, Saveetha Dental College, Saveetha Institute of Technical and Medical Sciences (SIMATS), Chennai, Tamil Nadu, India, between April 2024 to August 2024. This study received approval from the Scientific Review Board of Saveetha Dental College and Hospital (SRB/SDC/PERIO-2204/23/TH-151). For MTT analysis at various time intervals three groups were compared A2M Hydrogel, pure hydrogel (without A2M), control group (sterile water).

Study Procedure

Materials: Commercially available A2M from human plasma in lyophilised form was sourced from Sigma-Aldrich. Pluronic F127 (a synthetic hydrogel composed of ethylene oxide and polypropylene oxide copolymers) and chitosan (a natural polysaccharide serving as the base material for the hydrogel) were also used. Other reagents included 1% acetic acid for dissolving chitosan and Phosphate-Buffered Saline (PBS) for protein dissolution and pH adjustment.

Preparation of A2M-based hydrogel: To prepare the polymer base, 30 g of Pluronic F127 was dissolved in 100 mL of deionised water and stirred at room temperature for two hours using a magnetic stirrer until a clear, homogeneous solution was achieved. Concurrently, a 2.5% chitosan solution was prepared by dissolving 2.5 g of chitosan in 100 mL of 1% acetic acid, followed by stirring for three hours to ensure complete dissolution. The two solutions were then mixed in a 1:1 ratio and stirred overnight to form a uniform blend [Table/Fig-1] [13].

For drug incorporation, 1 mg of A2M was reconstituted in 500 μ L of deionised water to yield a 2 mg/mL A2M solution. Subsequently, 200 μ L of this A2M solution was added to 2 mL of the prepared polymer mixture. The fabricated hydrogel exhibits a unique thixotropic property, remaining in a flowable liquid state at 4°C and transforming into a solid gel at 37°C [Table/Fig-2].

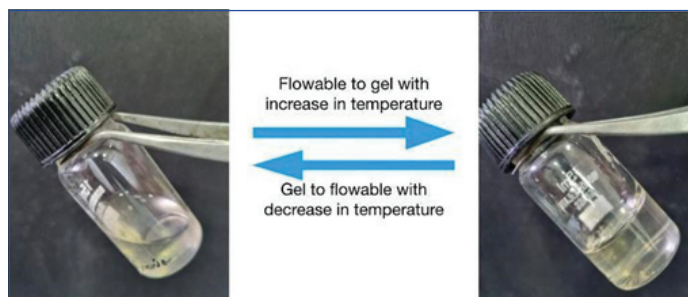
MTT assay methodology for cell viability evaluation: The cytotoxicity of the polymer base of the hydrogel (Pluronic F127 and chitosan) and the hydrogel loaded with A2M was analysed using the MTT assay, compared with a control group (sterile water) on Human Gingival Fibroblasts (HGFs). This assay is a colorimetric method used to determine cell viability by assessing the reduction of MTT to formazan crystals in metabolically active cells [14].

HGFs obtained from healthy donors were cultured in complete Dulbecco's Modified Eagle Medium (DMEM). The cells were kept at 37°C in a humidified incubator with 5% CO₂. At a density of 1×10^4 cells per well, the cells were seeded in 96 well plates and left to adhere overnight [Table/Fig-3].

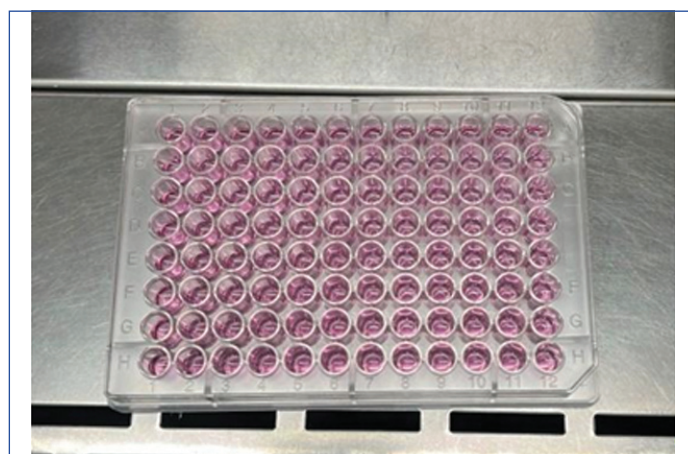
Following 24 hours of incubation, the cells were treated with one of the following groups: Control (untreated cells), pure hydrogel base



[Table/Fig-1]: Pluronic F127 and chitosan polymer base.



[Table/Fig-2]: Thixotropic nature of A2M hydrogel, flowable at 4°C and 37°C.



[Table/Fig-3]: Human Gingival Fibroblast (HGF) cells seeded in 96 well plates.

(Pluronic F127 + chitosan), or A2M hydrogel at varying concentrations of 25, 50, 100, 200, 300, and 400 μ g/mL to assess concentration dependent cytocompatibility. To evaluate the effects of prolonged exposure on cell viability, only the 50 μ g/mL concentration of A2M hydrogel and pure hydrogel was used, along with the control group. These groups were incubated and analysed on day 1, day 3, and day 5.

After each specified time point, the medium was aspirated, and the cells were rinsed with PBS. MTT solution (5 mg/mL in PBS) was added to each well to achieve a final concentration of 0.5 mg/mL. The plates were incubated for 4 hours at 37°C in the dark to allow metabolically active cells to reduce MTT to formazan crystals. Post-incubation, the MTT solution was removed, and 100 μ L of DMSO

was added to each well to dissolve the formazan crystals. The plates were gently shaken for 10 minutes to ensure uniform dissolution.

Absorbance was measured at 570 nm using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek, Winooski, VT, US). Cell viability (%) was calculated based on the absorbance ratio of treated samples to the control. The morphology of viable cells was observed under a phase-contrast and fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) at 20× magnification.

$$\text{Cell viability (\%)} = \frac{\text{OD (test sample)} - \text{OD (blank)}}{\text{OD (control)} - \text{OD (blank)}} \times 100 \quad [15]$$

Drug release analysis: To investigate the drug release profile of the α 2M hydrogel, a concentration of 50 micrograms/mL of α 2M was used. Three mL of the hydrogel was placed inside a dialysis membrane and submerged in 100 mL of phosphate buffer solution (PBS, pH 7.4), as illustrated in [Table/Fig-4]. At predetermined time intervals (150, 300, 450, and 600 minutes), 2 mL of the releasing media were withdrawn. The withdrawn samples were then analysed using a spectrophotometer (Jasco V-730, UV-Spectrophotometer) to quantify the released drug. This process allowed for the determination of cumulative drug release over time, helping to evaluate the release kinetics of the hydrogel [16].



[Table/Fig-4]: A2M Hydrogel inside dialysis membrane submerged in PBS for drug release analysis.

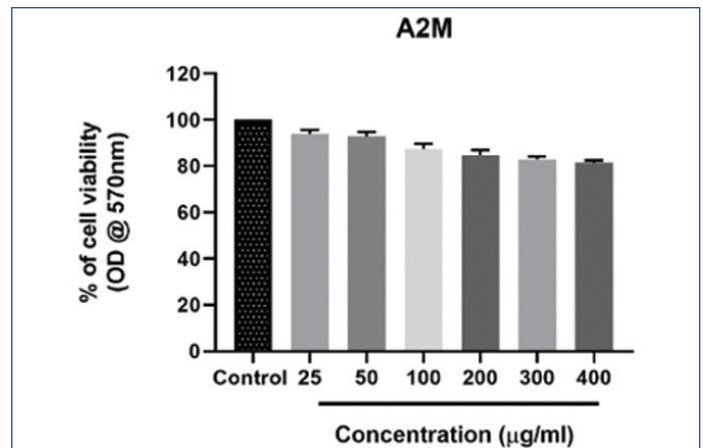
STATISTICAL ANALYSIS

Data were analysed using SPSS, version 23.0. Intergroup comparisons of fibroblast viability at day 1, day 3, and day 5 among the control, pure hydrogel, and A2M hydrogel groups were performed using one-way ANOVA, followed by Tukey's post-hoc test for pairwise analysis. A p-value of ≤ 0.05 was considered statistically significant.

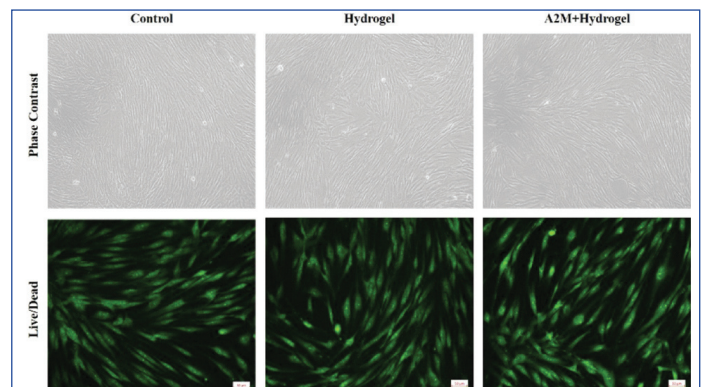
RESULTS

MTT assay of A2-M hydrogel at various concentrations: The cytocompatibility of α 2M was evaluated in HGFs at concentrations of 25, 50, 100, 200, 300, and 400 micrograms/mL. At 25 and 50 micrograms/mL, cell viability remained above 90%, which is almost equivalent to the control group, indicating good cytocompatibility. At other concentrations, cell viability at an optical density of 570 nm was more than 80%.

The assay revealed that concentrations of A2M from 25 to 400 micrograms/mL were deemed optimal for LDD [Table/Fig-5], as they maintained $>80\%$ cell viability and healthy cell morphology. Phase-contrast images across all groups revealed elongated fibroblast morphology, indicating the absence of cytotoxic effects. The live/dead fluorescence assay showed strong green fluorescence in all groups, confirming high cell viability with negligible red fluorescence, signifying minimal cell death. The A2M hydrogel group displayed cell density comparable to or slightly greater than the control, highlighting its excellent cytocompatibility and potential to support cell proliferation [Table/Fig-6].



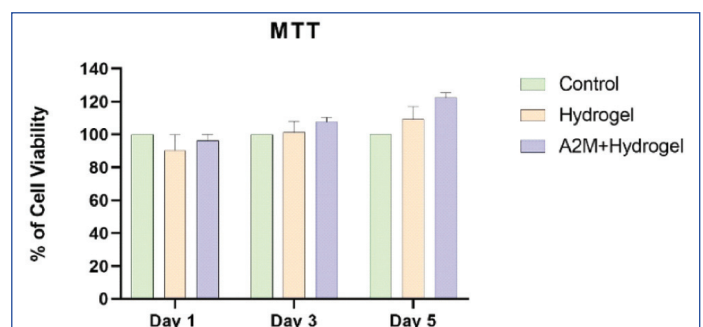
[Table/Fig-5]: Showing cell viability of A2M hydrogel 95% at 25 µg/mL, 93% at 50 µg/mL, 90% at 100 µg/mL, 87% at 200 µg/mL, 85% at 300 µg/mL, and 82% at 400 µg/mL.



[Table/Fig-6]: Cell viability and morphology of Human Gingival Fibroblasts (HGFs) under different treatment conditions: Control, Pure Hydrogel, and A2M+Hydrogel.

MTT analysis for cell viability of A2M hydrogel at various time intervals:

The biocompatibility of A2M hydrogel was assessed by evaluating its effects on HGFs using the MTT assay. Cells were treated with A2M hydrogel and pure hydrogel compared with the control for 24, 72, and 120 hours (day 1, day 3, day 5). At day 3 and day 5, A2M hydrogel showed increased cell viability, depicting cell proliferation and the regenerative capacity of A2M with no significant morphological changes, indicating excellent biocompatibility [Table/Fig-7].



[Table/Fig-7]: showing MTT assay of A2M hydrogel, Pure hydrogel and control group (sterile water) at day 1, day 3, day 5.

[Table/Fig-8] illustrates that on day 1, all groups showed comparable cell viability, indicating no cytotoxic effects. By day 3, the A2M hydrogel

| Optical Density (OD) reading at different durations | A2M hydrogel (Mean±SD) | Pure hydrogel (Mean±SD) | Control | p-value |
|---|------------------------|-------------------------|---------|---------|
| Day 1 | 99±21 | 98±32 | 100±11 | 0.36 |
| Day 3 | 110±45 | 105±36 | 100±21 | 0.04* |
| Day 5 | 122±52 | 110±4 | 100±21 | 0.001 |

[Table/Fig-8]: Comparison of viability of fibroblasts at day 1, day 3 and day 5 between A2M hydrogel, pure hydrogel and Control groups.

*statistically significant

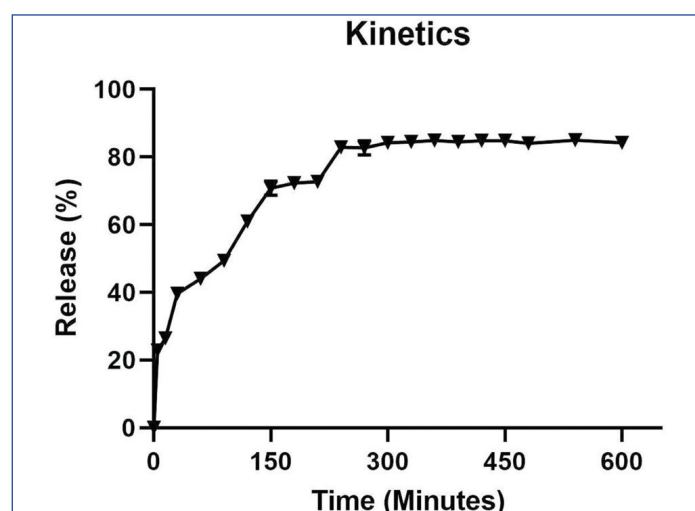
group exhibited a notable increase in cell proliferation ($p < 0.05$). This effect became more pronounced by day 5, with the A2M+hydrogel group showing the highest cell viability ($p < 0.05$), highlighting its strong potential to support cell growth and tissue regeneration.

[Table/Fig-9] shows that on day 1, all groups exhibited similar levels of cell viability, indicating that both the pure hydrogel and A2M-loaded hydrogel were biocompatible. By day 3, and more notably on day 5, the A2M+hydrogel group demonstrated a significant increase in cell viability compared to the control and pure hydrogel groups. This gradual improvement highlights the ability of the A2M hydrogel to enhance fibroblast proliferation over time.

| Time | Groups | Mean difference | p-value |
|-------|-------------------------------|-----------------|---------|
| Day 1 | A2M hydrogel vs pure hydrogel | 0.1 | 0.47 |
| | Pure hydrogel vs control | 0.7 | 0.12 |
| | A2M hydrogel vs control | 0.3 | 0.23 |
| Day 3 | A2M hydrogel vs pure hydrogel | 1.7 | 0.08 |
| | Pure hydrogel vs control | 1.5 | 0.1 |
| | A2M hydrogel vs control | 5.8 | 0.001 |
| Day 5 | A2M hydrogel vs pure hydrogel | 5.4 | 0.001 |
| | Pure hydrogel vs control | 4.6 | 0.01* |
| | A2M hydrogel vs control | 7.2 | 0.00* |

[Table/Fig-9]: Tukey's post-hoc pairwise comparisons for fibroblast viability.
*statistically significant

Drug release analysis: The drug release profile of the $\alpha 2$ macroglobulin (50 micrograms/mL)-encapsulated Pluronic F127 and chitosan-based hydrogel, observed over a 600-minute period, reveals a distinct biphasic release pattern. In the initial phase, there is a rapid burst release, during which approximately 20% to 40% of $\alpha 2$ -macroglobulin is released. This early release is likely attributed to the diffusion of drug molecules situated near the surface or in loosely bound regions of the hydrogel matrix. Following this initial phase, the release enters a more gradual and controlled stage, during which an additional 40% of the drug is released over the next 450 minutes. This sustained release is primarily governed by the diffusion of $\alpha 2$ -macroglobulin through the internal hydrogel network and its interactions with the matrix components. By the end of the 600-minute duration, the cumulative release reaches approximately 80% to 90%, indicating near complete liberation of the drug. This biphasic release profile underscores the potential of the hydrogel system to deliver both an immediate and a prolonged therapeutic effect [Table/Fig-10].



[Table/Fig-10]: Drug release pattern of A2M hydrogel at various time intervals.

DISCUSSION

This study aimed to develop and evaluate an A2M-loaded hydrogel for LDD in the management of periodontitis. A2M, a broad-spectrum plasma protease inhibitor with host-modulatory properties, was

incorporated into a hydrogel matrix composed of Pluronic F127 and chitosan to enhance its clinical potential in periodontal therapy. The formulated hydrogel demonstrated favorable biocompatibility and exhibited a biphasic drug release profile in-vitro, supporting its suitability as a localised therapeutic system.

The biocompatibility of the A2M hydrogel was assessed using the MTT assay on HGFs. Results showed that cell viability remained above 80% across a concentration range of 25-400 $\mu\text{g/mL}$, indicating low cytotoxicity.

Notably, concentrations of 25 and 50 $\mu\text{g/mL}$ were comparable to the control group, affirming the hydrogel's safety for periodontal applications. These findings are consistent with previous literature suggesting that protein-based hydrogels, particularly those composed of natural and synthetic polymers, are well tolerated by oral soft-tissue cells [17,18]. In addition to maintaining cell viability, the A2M hydrogel also supported fibroblast morphology and proliferation over a period of five days. Cells retained a healthy spindle shape with no evidence of necrosis or morphological abnormalities, further confirming its biocompatibility. This is critical for periodontal regeneration, where cellular health and function are essential to tissue repair; these findings are in accordance with previous studies [19].

The drug release profile of the hydrogel exhibited a biphasic pattern: an initial burst release within the first 150 minutes, followed by a sustained release phase extending up to 600 minutes, with cumulative drug release reaching approximately 90%. This behavior is ideal for periodontal therapy, where an early high concentration helps control acute inflammation, followed by prolonged drug presence to manage chronic microbial insult and modulate host response. The release kinetics were likely influenced by the polymer blend and crosslinking density, as well as physicochemical interactions between A2M and the matrix [20,21].

Compared to conventional therapies such as systemic antibiotics or mechanical debridement alone, the A2M hydrogel presents several potential advantages. Its host-modulatory effect targets the underlying inflammatory mechanisms of periodontitis rather than just the microbial component. Moreover, the thixotropic property of the hydrogel, which allows easy syringe delivery in liquid form and solidification at body temperature, makes it practical for clinical application in periodontal pockets.

Limitation(s)

Limitations of the study include its in-vitro design, which does not fully replicate the complex in vivo periodontal environment. The effect of enzymes, immune cells, and dynamic pH and temperature changes within the periodontal pocket may influence hydrogel performance differently. Additionally, long term effects on regeneration and inflammation control were not assessed.

CONCLUSION(S)

The present in-vitro study demonstrated that the A2M hydrogel prepared using Pluronic F127 and chitosan exhibited good biocompatibility with HGFs, maintaining cell viability above 80% at concentrations up to 400 $\mu\text{g/mL}$. The hydrogel showed a biphasic drug release pattern, with an initial burst release followed by a sustained release phase over 600 minutes. These findings indicate that the A2M hydrogel is a biocompatible and effective local drug delivery (LDD) system capable of controlled release under laboratory conditions. Further studies are required to evaluate its clinical efficacy. Future directions should include in-vivo evaluation of the A2M hydrogel in experimental periodontitis models to confirm its regenerative potential and anti-inflammatory efficacy. Studies should also explore combining A2M with antimicrobial or osteogenic agents to enhance multifunctional therapeutic outcomes. Optimisation of hydrogel concentration, mechanical properties, and biodegradability will also be necessary for clinical translation.

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PLAGIARISM CHECKING METHODS:

- Plagiarism X-checker: Dec 17, 2024
- Manual Googling: Jul 08, 2025
- iThenticate Software: Jul 12, 2025 (11%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. No

Date of Submission: **Dec 15, 2024**

Date of Peer Review: **Feb 10, 2025**

Date of Acceptance: **Jul 14, 2025**

Date of Publishing: **Aug 01, 2025**