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Anatomical and Histological Evaluation of Novel PLA-Keratin Bio-Composite Scaffolds for Tissue Engineering Applications

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ABSTRACT

Keratin, a natural protein extracted from poultry feathers, has gained significant interest in biomedical applications due to its biocompatibility, bioactivity, and ability to support cell growth. However, its large-scale utilization is limited by extraction challenges. In this study, keratin was successfully extracted from chicken feathers using a reductive method involving sodium metabisulfite, SDS, and urea, followed by dialysis and lyophilization. Biocomposite scaffolds were fabricated by solvent casting using polylactic acid (PLA) and varying concentrations of keratin. Subcutaneous implantation of PLA and PLA-keratin composites was performed in Sprague Dawley rats for 7, 14, and 28 days to assess biocompatibility and biodegradability. Histological analyses showed minimal inflammation and favorable tissue integration in all groups, with PLA-keratin scaffolds exhibiting increased cellular infiltration without adverse responses. The addition of keratin did not significantly alter fibrous tissue encapsulation but enhanced cellular response, indicating promising potential for tissue engineering. This simple and scalable method for keratin extraction and scaffold fabrication offers a viable approach to developing biodegradable, bioactive materials combining the mechanical strength of PLA with the biological functionality of keratin.

Key words: Keratin, Chicken feathers, Polylactic acid (PLA), Biocomposite scaffold, Tissue engineering, Biocompatibility, Subcutaneous implantation

INTRODUCTION

Keratin-based materials have gotten extraordinary attention because of their interesting attributes. In addition, because of its characteristic capability in progressing cell development, along side its capacity to epitomize both hydrophobic and hydrophilic medications, keratin has been progressively adopted for developing a wide range of bio-medical devices, particularly in the field of tissue engineering and drug delivery. Extraction of keratin from minimal expense biomasses from poultry industry is a difficult interaction hampered by the presence of a high substance of disulphide bonds that present the protein with high protection from chemical compounds, enzymatic and thermal treatment. Along these lines, the huge scope utilization of keratin emphatically relies upon the improvement of practicality and time-productive extraction techniques (Wang *et al.,* 2016).

The methods of extracting feather-keratin have been based generally on the breakage of disulfide bonds in keratin molecules by oxidation or reduction, with the combination of the use of dispersing reagents(Sharma *et al.,* 2019).

Biodegradable polymers are the chosen materials for scaffolding in tissue engineering. Synthetic biodegradable polymers provide excellent mechanical characteristics. Consequently, integrating them with natural polymers offers a benefit, as they create effective platforms with sufficient biocompatibility and appropriate mechanical properties for tissue engineering. Poly Lactic Acid (PLA) combined with keratin can be employed in applications necessitating stiffness. Solvent casting is a highly economical method that may be readily scaled to meet specific requirements. PLA -dissolved in chloroform is homogenized with lyophilized keratin powder in a homogenizer and was put on Petri plate to generate a thin bio-composite film once the volatile solvent evaporated. Subcutaneous implantation was carried out to determine the biocompatibility and biodegradability of materials implanted for lengths of 7, 14, and 28 days.

Qualitative and semi-quantitative analyses were conducted by evaluating the stained sections to assess the magnitude of the inflammatory response, the development of granulation tissue or fibrous tissue encapsulating the implant, and alterations in

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the implant, including fragmentation and infiltration by fibrous vascular tissue.

This study seeks to provide a framework utilizing synthetic and natural polymers, eliminate their constraints, and facilitate the generation of well-characterized materials with all requisite qualities for tissue engineering applications.

MATERIALS AND METHODS

After being cleaned and defatted using petroleum ether, clean chicken feathers were ground in a hammer / shearing mill. The milling process developed in two phases. Initially, coarse milling was conducted with a bigger shearing or hammer mill, subsequently followed by processing in a smaller mill for production of fine powder. The collected powder was subjected to sieving using a 25µm laboratory sieve, and the sieved particles were gathered and washed multiple times with petroleum ether to eliminate fat, utilizing a magnetic stirrer; the petroleum ether was subsequently separated using Whatman grade 1 filter paper. The powder contained feather particles measuring between 11 and 25 µm. The purified short feathers (7 g) were submerged in 250 ml of an aqueous solution comprising 8.0 M urea, 4.2 g SDS, and 0.2 M sodium metabisulfite, and continuously agitated at 65° C for five hours. The extracted solutions were subsequently filtered through a nylon mesh. The filtrates were then dialyzed in a dialysis tube (MWCO 6000–8000 Da) using distilled water for 3 days, with the distilled water being replaced three times daily. The solutions were then concentrated utilizing a rotary evaporator. The keratin was subjected to freeze-drying until its weight stabilized. This method of keratin extraction was described by Ayutthaya *et al.* (2015).

Polylactic acid (PLA) was dissolved in AR-grade chloroform in an enclosed container using a magnetic stirrer and poured onto to Petri plate to form a thin film. This Petri plate was then allowed to dry in a clean chamber for several hours to obtain a thin film.

Dissolved PLA was mixed with lyophilized keratin powder in a magnetic stirrer and poured onto a petri plate to form a thin film. This petri plate was then allowed to dry in a clean chamber for several hours to obtain a thin film.

Films intended for implantation studies were sterilized with 70% (v/v) ethanol for 24 h and followed by washing with Phosphate Buffer Saline (PBS) several times before the implantation studies.

Subcutaneous implantation of seventy-two Sprague Dawley rats (~ 250 g) was employed to evaluate the biocompatibility and biodegradability of materials that were subcutaneously implanted for 7, 14, and 28 days.

The animals were anaesthetized, shaved, and disinfected. After surface disinfection of the skin with iodine, a skin incision was created, and a tissue pocket was formed with blunt dissection. One alcohol-sterilized bio-composite of approximately 1.00 cm^2 was placed inside the subcutaneous pocket approximately 5 mm away from the cut, and the incision was sutured with surgical staples under sterile conditions(Figure. 1).

At the selected post-surgery time points, the rats were euthanized, with excess anaesthesia. The surface of the skin at the implantation site was photographed. The inner side of the skin attached to the implant was dissected out, and pictures were taken to assess the materials. Dorsal skin with an implant along the tissue underneath was taken out and processed.

The tissue samples were fixed in 10% neutral buffered formalin (NBF) immediately after collection. Once the fixation was achieved, the tissues were processed for paraffin block preparation by dehydration, clearing and impregnation to tissue section for histological investigation (Luna, 1968). The sections of 5-7 µm thickness were obtained on clean glass slides with rotary microtome. The paraffin sections were stained with Hematoxylin and Eosin stain to study the histomorphological details. For the qualitative and semi-quantitative studies, examination of the stained sections was performed to determine the extent of the inflammatory response, formation of the granulation tissue/fibrous tissue capsule around the implant, and changes occurring in the implant including fragmentation and penetration by fibrous-vascular tissue.

The assessment of implant material was performed as the method described by Royals *et al.*(1999). The first scale ascertained the presence and level of fibrous connective tissue, and the second qualified the inflammatory cell infiltrate response at the implant site.

Table 1: The grading scale (histological score)for connective tissue

Score	Criteria for Score
0	No difference from normal control tissue, no presence of connective tissue at or around the implant site
1	Presence of delicate spindle-shaped cells or mild fibroplasia
2	Presence of moderate connective tissue
3	Disruption of normal tissue architecture and presence of moderately dense fibrous connective tissue
4	Severe deposition of dense collagenous/connective tissue around the implant.

Table 2: The grading scale for the observed inflammatory cell infiltrate response

Score	Criteria for Score
0	No difference from normal control tissue, no presence of macrophages, foreign body giant cells, lymphocytes, eosinophils, or neutrophils at or around the implant site
1	Presence of a few lymphocytes or macrophages, no presence of foreign body giant cells, eosinophils, or neutrophils
2	Presence of several lymphocytes, and macrophages, with a few foreign body giant cells and small foci of neutrophils
3	Presence of large numbers of lymphocytes, macrophages, and foreign body giant cells, also no- table presence of eosinophils and neutrophils
4	Severe cellular infiltrate response to implant followed by tissue necrosis at or around the site.

RESULTS AND DISCUSSION

Ayutthaya *et al.* (2015) investigated keratin extraction from chicken feathers using various concentrations of sodium metabisulphite. This method uses short, clean feathers mixed with sodium-metabisulphite solutions ranging from 0 to 0.5 M. In Ayutthaya's paper, the results showed that the keratin percentage yield went from 36.2% to 87.6% as the concentration of sodium metabisulphite increased concentration of upto 0.2 M. Following this procedure, 71.5% of the keratin was extracted in the present study.

A sodium metabisulphite solution with a concentration of 0.2 M yielded keratin with a molecular weight range of 12-20 kDa. Because most of the keratin disulfide bonds are broken when the sodium metabisulphite concentration is above 0.2 M, the keratin percentage yield goes down. This is because short chains of keratin break free during dialysis.

Sinkiewicz and Ayutthaya reported extraction yields of 62.9% and 60.2%, respectively, using 0.5 M sodium metabisulphite (Ayutthaya *et al.,* 2015; Sinkiewicz *et al.,* 2017).

The keratin obtained had a molecular weight of around 10kDa and was ascertained through gel electrophoresis; in concurrence with the above authors.

The feather keratin is more stable and can remain stable in extreme pH. It can re-cross link the cysteine groups by oxidative coupling. The biomaterials formed from keratin are stable and can survive for months (Silva *et al.,* 2014) and for this desired quality, it was decided to couple this natural polymer with a synthetic polymer to overcome the drawback of the latter in isolation.

This study aimed to create a framework from synthetic and natural polymers, remove their limitations, and enable the production of highly characterized materials with all the properties required for tissue engineering applications.

Different concentrations of lyophilized keratin were added to PLA polymer when diluted with Chloroform. Among those, 25% and 50% Purified gave satisfactory results. 75% lyophilized keratin to PLA with solvent failed to form the sheet.

PLA with keratin (50%) was dissolved in 20 ml Chloroform for 30 min at room temperature and casted on the Petri plate to obtain a thin film after evaporation of Chloroform. A robust, grainy, homogeneous sheet exhibiting significant strength yet brittleness (little to no elasticity) was made.

This study was noteworthy due to the straight forward method of pouring the polymer with the solvent onto a Petri plate and permitting the solvent to evaporate, resulting in a polymer sheet characterized by porosity and high tensile strength. However, the addition of keratin reduced porosity, likely due to alterations in surface tension. The mixing efficiency was suboptimal, resulting in an uneven distribution of lyophilized keratin within the synthetic polymer PLA, which caused the formation of spherical protein islets on the surface.

PLA has a broad spectrum of applications, but there are certain limitations such as i) slow degradation rate, which could be up to years, ii) hydrophobicity leading to low cell affinity, and can elicit, in some cases, an inflammatory response from the living host upon direct contact with biological fluids. iii) brittleness, with less than 10% elongation at break, and iv) Lack of reactive side-chain groups - chemically inert with no reactive side-chain groups makes its surface and bulk modifications challenging. PLA is thermally unstable and exhibits rapid loss of molecular weight and consequent erosion of its mechanical properties as well. (Farah *et al*., 2016)

Blending PLA with other polymers offers

convenient options to improve associated properties or to generate novel PLA polymers/blends for target applications. Good solvents for PLA products are dioxane, acetonitrile, chloroform, methylene chloride, 1,1,2-trichloroethane, and dichloroacetic acid (Ebrahimi and Ramezani, 2022)

Implantation Studies

Biocompatibility was evaluated by subcutaneous implantation of scaffold in six Sprague Dawley rats from each group. Group 1 rats were studied after one week, Group 2 after the second week, and Group 3 after four weeks of subcutaneous implantation.

***In situ* Gross observation**

All rats survived throughout the study period. The subjects exhibited no complications and appeared unharmed by the implants. All implants were located at the site of the initial transplantation (Figure 2). In order to demonstrate scaffold integration, visible capillaries were evaluated (Figure 3).

Macroscopic evaluation showed an intact structure for scaffolds inserted. The tissue was found to be integrated into the host tissue. Slight hyperaemia was evident in all the groups.

Evaluation of the *in vivo* host tissue reaction to polymer implants with and without keratin:

Histomorphology

Hematoxylin and eosin staining revealed cellular infiltration and encapsulation surrounding the scaffold. Elevated levels of neovascularization and heightened recellularization around the inflammatory cells were observed across all groups (Figure. 4).

Encapsulation or weak inflammatory reaction was expected around film materials in the beginning stage in beginning stages after implantation. On the other hand, the tissues surrounding the implant were devoid of inflammatory cells and gradually became invaded by host cells. This indicates the physiological response to unfamiliar material and attempt to confine it.

The H&E staining of subcutaneous implants and the skin revealed little to no inflammatory cells in the implants and adjacent tissues, indicating favourable biocompatibility of keratin containing biomaterial.

No statistically significant variations were observed between weeks 1 and 4 of the implantation studies.

Connective tissue covered the scaffold *in vivo*. A microphotograph of the scaffolds with conventional H&E staining revealed that after 2 weeks of *in vivo*, a multi-layer epithelium was present on top of the scaffold; after 4 weeks, these layers thickened all around the scaffold in all the groups.

A multilayered connective tissue delineated the scaffold from the surrounding subcutaneous region.

The biocomposites exhibited no comparable levels of inflammation, regardless of the presence of keratin in the scaffolds. Nonetheless, the study did not aim to evaluate the degradation levels post-implantation.

All *in vivo* transplants were accepted and integrated into the surrounding tissues, and successfully demonstrated cell proliferation *in vivo*. No adverse effects were observed, including transplant expulsion or any inconveniences exhibited by the rats.

Poly L Lactic Acid (PLA) and PLA with keratin films: Poly L Lactic Acid (PLA) and PLA with keratin implants hadlesser thickness of connective tissue. Likewise, the thickness remained almost constant throughout 28 days. Addition of keratin to PLA did not cause any increase in the thickness of connective tissue encapsulation but did cause increase in cellular infiltration.

Tissue scaffolds generally need porous materials presenting interconnected pores with sizes from the nano to the microscale. Engineered

Table 3: The grading scale (histological score) for connective tissue was defined as follows:

Duration	PLA	PLA+Kr
7 days	1	1
14 days	1.6	1
28 days	1..3	1.6

Table 4: The grading scale for the observed inflammatory cell infiltrate response was defined as follows:

Duration	PLA	PLA+Kr
7 days	0.6	2.3
14 days	1	1.3
28 days	0.3	2.3

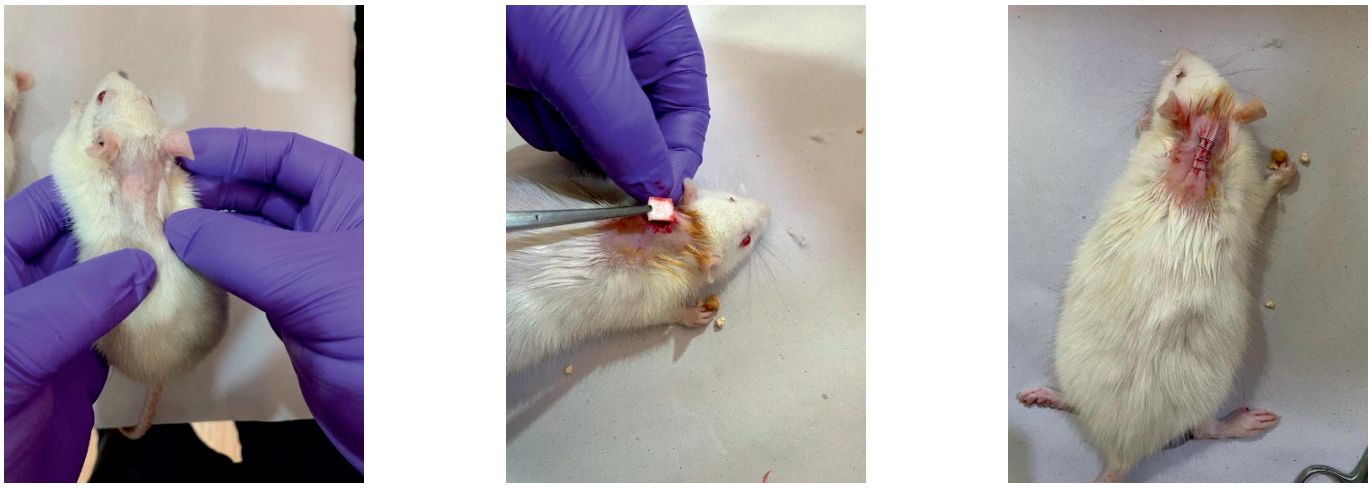

Fig 1. (a) Preparation of skin for implantation. (b) Implantation of polymer under the skin**.** (c) Skin is closed with a surgical stapler after implantation.

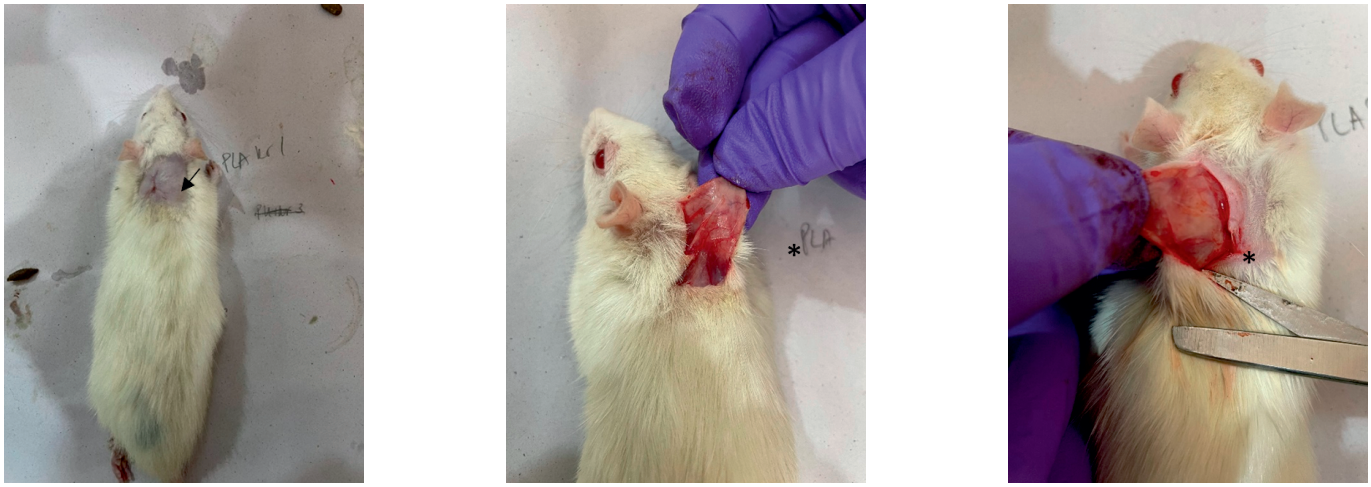

Fig 2. Gross observation of skin over implant material PLA and Keratin composite at one week with subcutaneous scaffold integration with surgical scar (arrow). **Fig 3.** Gross observation of implant material PLA implant (*) at (a) one week (b) four weeks with a fragile connective tissue covering and integrated implant material with no apparent characteristics of implant rejection.

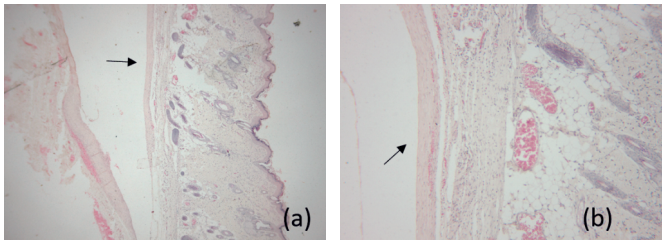

Fig 4. Photomicrograph of PLA implant along with skin under (a) Low power magnification (b) High power magnification- After 1 week of subcutaneous implantation, showing connective tissue covering-neoepithelium around the implant (arrow) under the dermis, with almost no inflammatory cells.

H & E 40X and 100X

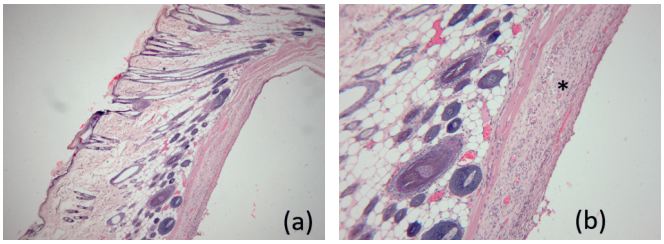

Fig 5. Photomicrograph of PLA + Kereatin implant along with skin under (a) Low power magnification (b) High power magnification- After four weeks of subcutaneous implantation- connective tissue (*) has no apparent increase in thickness and is devoid of chronic inflammatory markers or cells.

H & E 40X and 100X

scaffolds tend to mimic tissue microstructure and mechanical properties for enhanced cell proliferation and differentiation (Janik and Marzec, 2015).

Implantable bio-composites synthesized from this novel method produced non-harmful material with proven stability, biocompatibility, and mechanical strength. They have unique characteristics such as porosity, are ideal for drug and biological molecule binding, and are attributed

to keratin in practical methods.

Keratin biomaterials offer several advantages compared to conventional biomolecules, such as unique chemistry due to high sulfur content, excellent biocompatibility, ability for self-assembly, and inherent cellular recognition. As the properties of keratin biomaterials are further elucidated, controlled, and utilized, numerous biomedical applications will progress to clinical trials.

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