THE BIOLOGICAL ACTIVITY OF A NOVEL PEPTIDE BASED BIOPOLYMER FOR USING AS A BIOMATRIX

PROJECT SUBMITTED TO

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27.08.2018

This is to certify that Mr. Avijit Chakraborty, a 2nd year student, from Life Science and Biotechnology, Jadavpur University, had undergone summer project entitled "The Biological Activity of a Novel Peptide Based Biopolymer for Using as a Biomatrix" in my laboratory in June-August, 2018. He became familiar with biochemical techniques including culturing of mammalian cell lines, protein isolation, biochemical assays, Western blot techniques and methods involving polymerase chain reaction. Calm and co-operative by nature, he is sincere, motivated and has a sense of precision.

I wish him success in life.

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INTRODUCTION

Cell is a basic unit of life and all living organisms are alive because of this structural and functional unit. All life begins as a single cell and this is the basis for all the different biological studies whether it is knowing about the organelles and types of biomolecules present, its physiology or even its entire structural analysis, etc. For carrying out such biological studies the cells have to be obtained in an efficient manner. The property of cells to sustain when provided with appropriate conditions is utilized in *in-vitro* culturing of cells. The cells are cultured based on whether they are adherent or are culturable in suspension.

Since most of the mammalian cells are adherent, they require a surface or matrix for their adherence. Cell culture is an extremely widely used process by which cells are removed from their natural environment and grown artificially under controlled and monitored conditions. It is generally done *in-vitro* on glass or plastic materials. But the biocompatibility of matrices have always been a topic of debate along with the fact that most of the mammalian cells require very specific adhering sites and sequences for them to effectively attach and proliferate. Moreover the need of a tissue like environment for better and appropriate growth of the culturable cells have been of utmost importance as well, especially for regenerative studies and tissue based analysis. Hence came the need for development of biocompatible scaffolds for culturing such cells.

Development of biocompatible polymeric systems is of profound use both academically and commercially. These synthetic biomaterials have brought about immense progress in the field of tissue engineering and regenerative medicine.[1] - [3]. A promising approach for such biomaterials is their usage as scaffolds that mimic the mechanical and biophysical

properties of the Extra-Cellular Matrix (ECM) which acts by allowing cells to adhere and proliferate so as to regenerate the tissues and organs.[4] - [5]. The biomaterials are produced such that they effectively create that *in-vivo* like environment resembling the native ECM. Both synthetic polymers and peptide based materials are good enough to provide the scaffold but owing to the inherent biocompatibility and non-cytotoxicity the cell adhesive peptide cross-linked polymeric network are of major importance.[3], [6] - [9]Various peptide based synthetic ECM are prepared and utilized successfully for tissue engineering.[10] - [11]. However, linear peptides provide such surface for cell-adhesion through hierarchical supramolecular assembly only in most of the cases. On the other hand, in a cross-linked polymer, the network is an integral part of the polymer and attachment of cell-adhesive units on the network surface provide the desired surface for cell attachment and growth.

OBJECTIVES

The candidate is a peptide based biopolymer and for its effective application as a biological adherent matrix it must have appropriate adherent , non-cytotoxic and proliferative characteristics. Hence the biopolymer is to be checked for its biological properties. The biopolymer (in both the forms provided) is checked for by the following assays :

1. Cell Adhesion Assay
2. Cell Cytotoxicity Assay
3. Cell Proliferation Assay

1. The novel biopolymer is comprised of monomers which are itself comprised of two peptides linked to a compound CB[8] and complexed via various linkages and supramolecular interactions. Based on these interactions, two types of this biopolymeric candidate were provided, namely P1 and P2 and are checked for their ability to be used as

biologically safe biomatrices based on interactions with RAW 264.7 cells, a murine macrophage cell line. This cell line is used for different biological studies (monocyte/macrophage-like cell line RAW 264.7 has been one of the most commonly used myeloid cell line for more than 40 years) in murine models and is used in studies for the disease “Leishmaniasis” as well.

MATERIALS AND METHODS

MATERIALS NEEDED :

1.Supplied lyophilized candidate biopolymer

2.Chemicals, reagents and kits needed for the respective assays

3.RAW 264.7 cell line – A murine macrophage cell line. EXPERIMENTS PERFORMED :

Cell Adhesion Assay :

Cell adhesion property of P1 and P2 was assessed according to the method described in Yeo et al.,[12]which is as light modification of the cell count assay by crystal violet described by Kueng et al.[13] A 96-well ELISA plate was first U.V. cross-linked overnight with P1 and P2 of different working concentrations. Then the wells were blocked with 100 µL of 3% BSA in DMEM for 1 h. Then the wells were washed twice with DMEM containing 0.1% BSA. Next RAW 264.7 macrophage cells were resuspended in DMEM containing 0.1% BSA. 100 µL of the cell suspension containing 104cells were added to each well and incubated at 37 °C for 1 h in 5%CO2. Finally, the cells were stained with 0.5% Crystal Violet aqueous solution in 20% methanol for 10 min. The unattached cells were washed 3 times with deionized waters and visualized under NikonTs2R-FL microscope equipped with 40× objective. The images thus captured were processed by Image J program

downloaded from National Institute of Health and mounted using Adobe Photoshop Software. To measure quantitatively, 200 µL of0.1% SDS were added to each well to solubilize the stain and absorbance was measured at 570 nm. All assays were performed intriplicate. The cell adhesion was measured as the fold change in cell number (which is proportional to measured O.D.) with respect to control untreated cells.

Cytotoxicity Assay :

MTT assay was performed to see the effect of the polymer on cell viability. P1 and P2 were initially dissolved in deionized water and then diluted to prepare working concentrations of 10, 20, 40, 80, 160, 320, 640, and 1280 µM. A 96-well plate was UV cross-linked with different concentrations of P1 and P2 overnight. Then, 104 cells were grown in P1 and P2 coated 96-well plate and incubated for 24 h. Thereafter, MTT (5 mg/mL) was added to the plates and incubated at 37 °C for 4 h. Finally, formazan crystals were solubilized in solubilization buffer and absorbance was measured at 570 nm. All assays were performed in triplicate. The extent of cell viability was measured as the percent decrease in viability with respect to untreated cells.

Cell Proliferation Assay :

Raw 264.7 macrophage cells were plated at a density of 104cells/well in a 96 well P1 and P2 (10–1280µM) coated microplates (Tarsons). After 24, 48, and 72 h incubation in medium, cell proliferation was determined by using CyQUANT Direct Cell Proliferation Assay Kit (Thermo Fisher Scientific). Briefly, after incubation of cells, medium was removed and cells were incubated with CyQUANT reagent for 1 h at 37 °C according to manufacturer's instructions. Plates were analyzed by using a microplate reader (excitation 508 nm, emission 527 nm).

RESULTS

CELL-ADHESION :

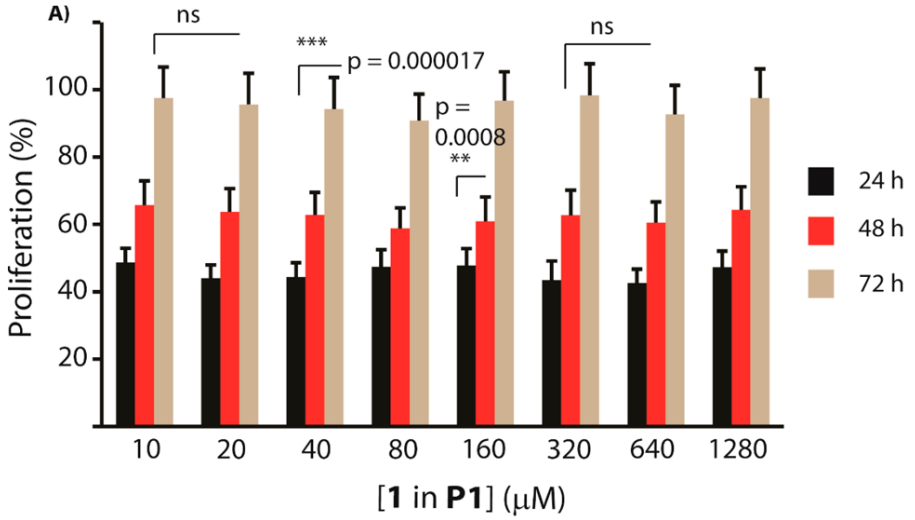

Figure 1(A) : RAW 264.7 Macrophage cells incubated with different concentrations of P1 and evaluated after 24, 48, and 72 hours of incubation. The result is representative of means ± SD of three independent experiments.

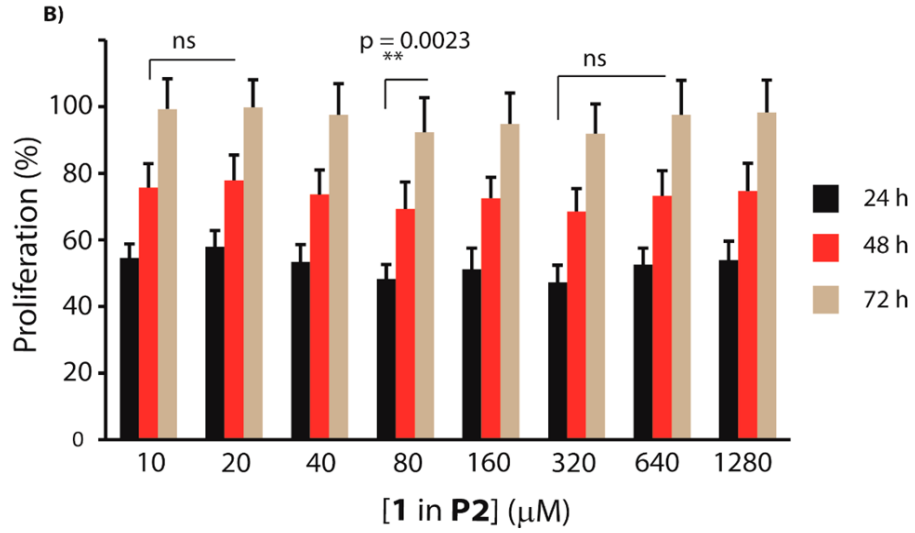

Figure 1(B) : RAW 264.7 Macrophage cells incubated with different concentrations of P2 and evaluated after 24, 48, and 72 hours of incubation. The result is representative of means ± SD of three independent experiments.

Eight working concentrations of P1 and P2were prepared with deionized water such as 10, 20, 40, 80, 160,320, 640, and 1280 µM and cell adhesion assay was performed(**Figure 1**). The fold changes were calculated with respect to the control cells. Up to 160 µM concentration of P1, a little hike in cell adhesion property of the polymer was observed. At 320 and 640 µM, 3-fold changes were recorded (with respect to the control). Dose-dependent gradual increase in cell adhesion property was also observed when RAW cells were incubated with P2. However, at 1280 µM concentration, polymer P1 showed greater cell adhesion property (9.38-fold as compared to control, $p < 0.0001$) than polymer P2 (6.4-fold over control, $p < 0.0001$). These results suggest that both P1and P2, at this concentration range, can bind cells significantly.

CYTOTOXICITY :

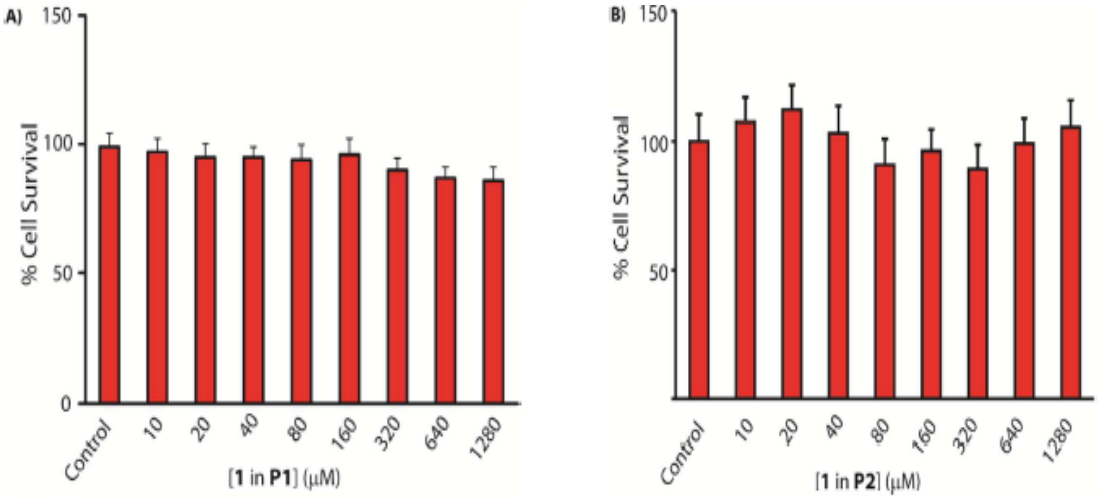

Figure 2 : Cytotoxic activity of **(A)** P1 and **(B)**P2 against RAW 264.7 Macrophage cells treated with different concentrations of the polymer for 24 hours and its effect on cell observed and measured by MTT cell viability assay.

The cross-linked polymers have surfaces decorated with RGDS sequence, which is well-known for its cell-binding properties. Cytotoxicity assay was done with both the polymers, P1 and P2. Different concentrations of P1 andP2 (10–1280 μM) were tested for cytotoxicity against RAW264.7 murine macrophage cell line (**Figure 2)**. No significant changes were observed in cell viability when cells were incubated with up to 1280 μM of P1 and P2 ($p > 0.03$)as ascertained by MTT assay. These results suggest that both the polymers P1 and P2 had no significant toxic effect on RAW 264.7 macrophage cells up to 1280 μM concentration.

EFFECT OF P1 AND P2 ON CELL PROLIFERATION :

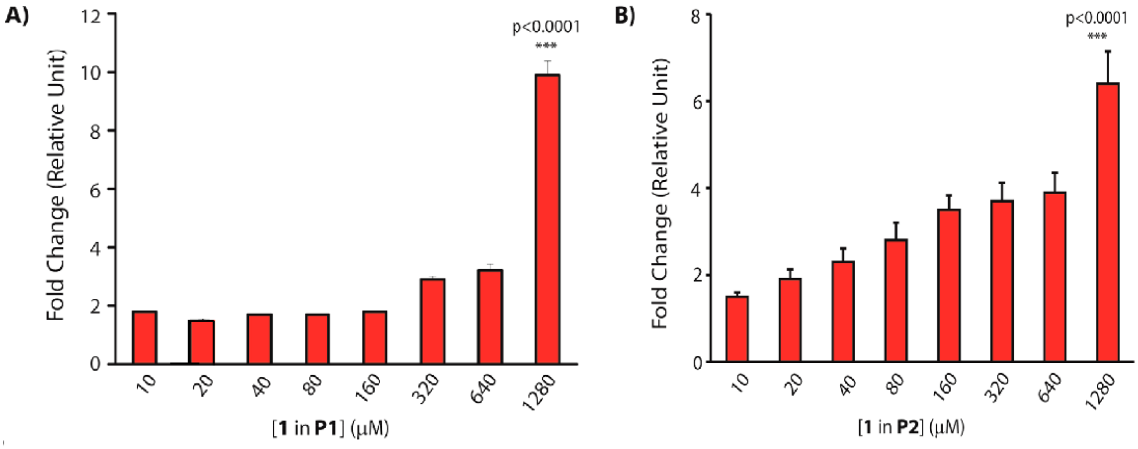

Figure 3 : RAW 264.7 Macrophage cells seeded on culture plates for 24 hours pretreated with different concentrations of **(A)**P1 and**(B)**P2 overnight. The result is representative of means ± SD of three independent experiments.

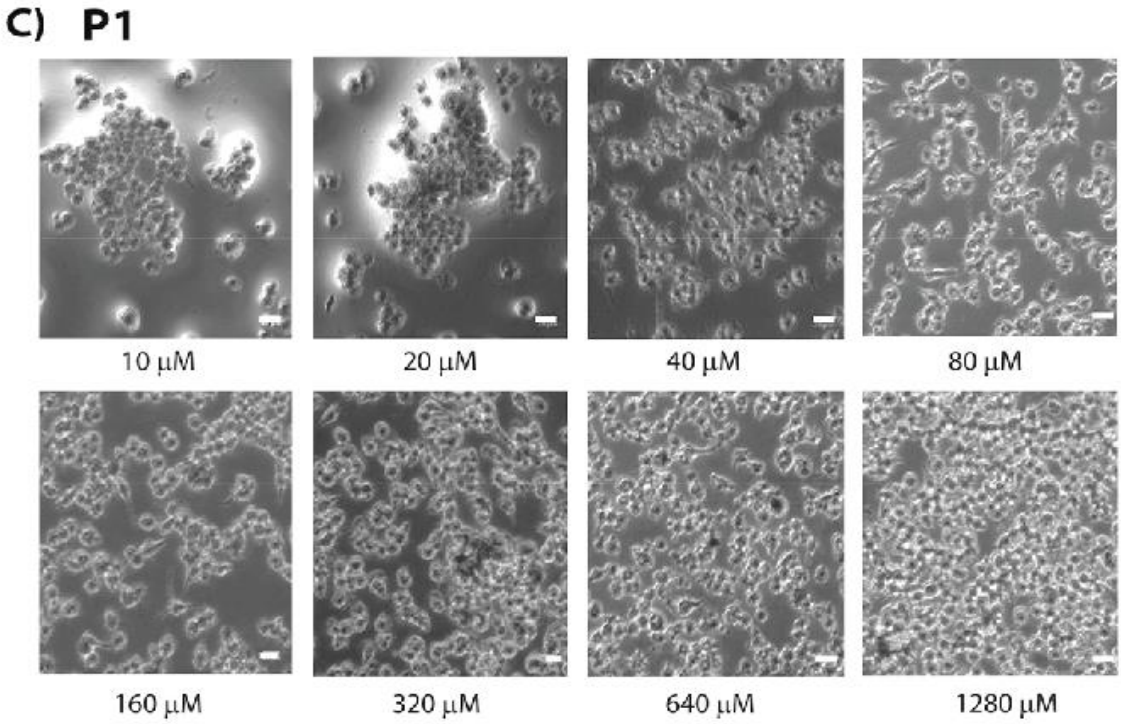

Figure 3(C) : Microscopic images of RAW 264.7 Macrophage cells adhering to P1 for different concentrations

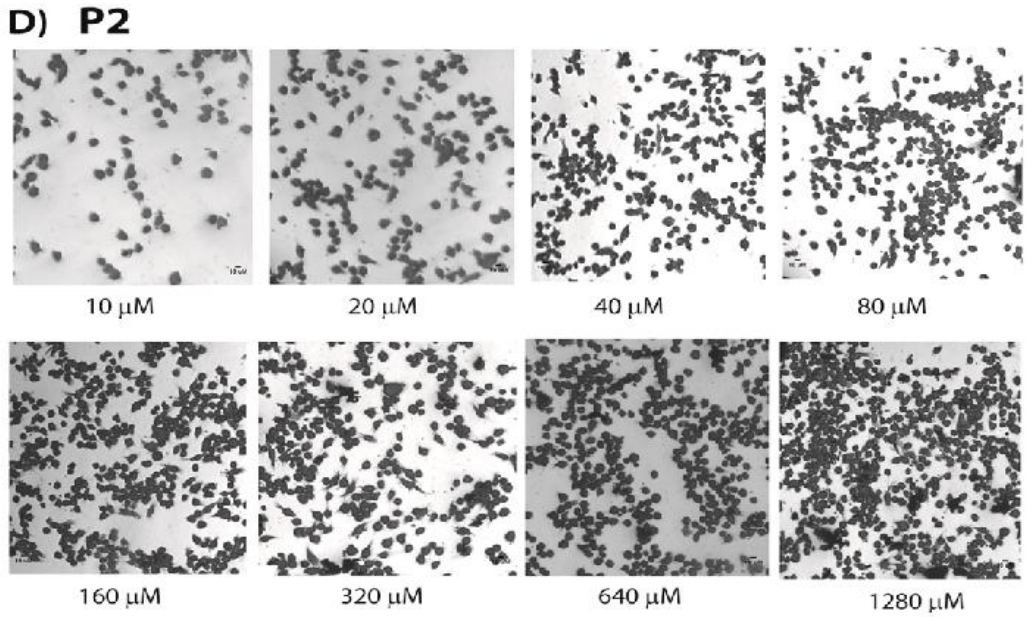

Figure3(D) : Microscopic images of RAW264.7 Macrophage cells adhering to P2 for different concentrations.

Cells were incubated with different concentrations of P1 and P2 and cell proliferation was measured after 24, 48, and 72 h using CyQUANT cell proliferation assay kit [**Figure 3 (A,B)**]. After 24h of incubation, no significant change in cell proliferation was observed when cells were incubated with up to 1280 μM peptide ($p > 0.02$). After 48 h, cellular proliferation increased by 36.81 ± 6% (for P1) and 40.15 ± 5% (for P2) on an average with respect to 24 h incubated cells, whereas 72 h incubation increased cellular proliferation by 52.4 ± 5% (forP1) and 33.5 ± 4% (for P2) on an average compared with 48h incubated cells. Different dosage of peptide was unable to make any significant changes in cell proliferation at a specific incubation time point. This result suggests that cells can proliferate in these polymer coated microplates. However, P1was found to be marginally better than P2 in this respect.

CONCLUSION

In summary, a new and simple approach is developed to create peptide based cross-linked polymer. The cross-linking of a small peptide is achieved through covalent disulfide bonds, supramolecular ternary complexes of CB[8], and HRP catalyzed Tyr dimerization. The size of the polymer can be tuned by changing the sequence of cross-linking. A pendent RGDS sequence at the surfaces of these polymers allowed to utilize the non cytotoxic cross-linked polymers for efficient adhesion and proliferation of RAW264.7 murine macrophages. The effective cell adhesion and proliferation at these polymer surfaces promises further application of this strategy to prepare artificial ECM. Animal model based therapeutics do not always reflect similar scenario in humans. Therefore, synthesizing ECM from cell adhesive sequence containing artificial scaffolds is a crying need. A similar approach can also be taken for use in other applications by modifying the sequence.

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