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Synthesis and Development of Some Biodegradable Polypeptides as Antimicrobial Agents

Shailendra K. Saraf[1]*, M. S. M. Rawat[2], Avinash C. Tripathi[1]

[1]Division of Pharmaceutical Chemistry, Faculty of Pharmacy, Babu Banarasi Das Northern India Institute of Technology, Lucknow-226028, U.P., India.
[2]Department of Pharmaceutical Sciences, H. N. B. Garhwal University, Srinagar-246174, Uttarakhand, India.

Abstract : Biodegradable and bioactive polymers are gaining considerable attention in the field of biomedical research to achieve therapeutic effects, drug-targeting and as drug carriers. Two acidic amino acid (glutamic and aspartic acids) monomer units were converted into polyanionic long chain polypeptides- poly-glutamic acid (PGA) and poly-aspartic acids (PAA). The synthesized derivatives were characterized by various physicochemical and spectral methods. The approximate molecular weight of the synthesized polypeptides were determined by Sorenson's method, and further confirmed by gel electrophoresis and MALDI-TOF techniques. The *in vitro* hydrolysis studies of the synthesized polypeptides was performed in simulated conditions outside the body, which mimic truly the *in vivo* scenario, and was used to study biodegradation. Results of the study clearly demonstrated that PGA and PAA are less prone towards hydrolytic degradation under acidic conditions in comparison to that in slight alkaline medium. Finally, the synthesized polypeptides were subjected to biological screening for antibacterial activity, at 10 and 50 µg/mL concentrations, against *P. morganii* and *S. aureus* bacterial strains using agar diffusion (filter paper disc) method. The antibacterial activity was exhibited by both PGA and PAA against both the selected strains, with the potency of PGA being slightly more than that of PAA. This could be attributed to the selective uptake of the compound by the particular strains of microorganisms. Thus, it can be concluded that polyanionic polypeptides can exert broad spectrum antimicrobial activity, and shall be biodegradable by physiological enzymes into smaller non toxic fragments/monomeric units having essential dietary value.

Keywords : Biopolymers, Drug-Polymer conjugates, Biodegradable polypeptides, Antibacterial, Polyglutamic acid, Polyascorbic acid.

Introduction

Recent years have seen a growing interest in biodegradable and bioactive polymers in biomedical applications to achieve therapeutic effects, drug targeting and as drug carriers. Biodegradable polymers degrade to release individual polymer molecules or smaller fragments during or after the drugs are released. Most of the bioactive polymers are poly-electrolytes which exert bioactivity, but the exact relationship between polymer structure and bioactivity is not well established. Thus, it may be difficult to find polymers with specific bioactivity. Nevertheless, the idea of using bioactive polymers as a part of biodegradable polymers presents new opportunities in the development of future drug delivery devices [1]. A large number of natural polyanions possess innate physiological properties alongside various synthetic polymers which elicit significant

bioactivities such as antitumor, antiviral and antibacterial [2, 3]. Polymers such as synthetic polyanionic electrolytes can express bioactivity directly, as polymeric drugs. Several synthetic polycarboxylic acid polymers have been evaluated for biological activities, such as stimulation of the reticulo-endothelial system, modulation of humoral and/or cell mediated immune responses and improvement of resistance to various microbial infections [4]. Polyanions are cytotoxic against both DNA and RNA pathogenic viruses from several major virus groups with diverse characteristics. The advantage of synthetic polyanions is their ability to protect mammalian hosts against a broad variety of viruses and bacteria, compared to the narrow antiviral/ antibacterial spectrum of most conventional drugs used for chemotherapy. Synthetic polyanions can also provide prolonged protection against viral and other microbial infections [5-7]. These activities of polyanions are affected by the structure and molecular weight of the polymers. The molecular weight of the polymer should not exceed 50,000 to allow for excretion from the host, thus preventing long term toxicity. On the other hand, low molecular weight polyanions (<30,000) were shown to be ineffective against these infections [8]. The mechanism of cell interaction with high molecular weight polymers has been studied from the standpoint of polymeric prodrugs, where the polymer is the carrier. The behaviour of prodrugs or drug conjugates in the body is determined by physicochemical properties of the prodrugs as well as by the biological environment [9]. Normal cells are permeable to polymers depending on their molecular weight, with a drastic change around 30,000. However, the tissue inflammation produces enhanced membrane permeability with the subsequent leakage of proteins and lipids from the blood vessels into the interstitial space [10]. In many cases, the use of drugs with a high therapeutic potential is restricted due to severe side effects. Systemic drug toxicity can be significantly reduced by the site directed delivery of drug which limits the activity to the location of the disease. The chemical conjugation of drugs to macromolecules presents an efficient way of modifying pharmacological and biochemical characteristics of the drugs [11]. To date, advances have mostly been made in the studies of biodegradable polymers/ hydrogels, which are mostly, drug loaded systems. Such systems inadvertently pose problems in formulation, dose regulation and generate metabolic products with untoward effects. The literature survey reveals that not much work has been done on polymers that can have biological activity by themselves. Such polymers may be comprised of units, which themselves are parts of various physiological processes. Also, these polymers may be utilized to synthesize drug-polymer conjugates as prodrugs or latentiated derivatives. The polymer backbone is modified as to get the desired hydrophilicity/ hydrophobicity, site specificity and such physical, pharmacological and biochemical characteristics.

The present study envisages to synthesize and evaluate polymers of glutamic acid and aspartic acid and to investigate: Whether, such polymers possess any biological activity (antimicrobial) by themselves; to what extent the physical properties of polymer vary with structure; to what degree the *in vitro* cleavage rate vary with structure and; to suggest the probable formulations for the polymers.

Materials and methods

Procurement and characterization of amino acids

Amino acids, glutamic acid and aspartic acid were procured from E. Merck KG and were subjected to following tests for identification and characterization.

Melting point: Glutamic acid: 160°C, aspartic acid: 271°C.

Solubility profile: Solubility studies were conducted in solvents- water, ether, methanol, salt solution, dilute hydrochloric acid and dilute sodium hydroxide. Results are given in Table 1.

Table 1: Solubility profile of selected amino acids.

S. N.	Solvent	Solubility	
		Glutamic acid	**Aspartic acid**
1.	Water	Soluble	Soluble
2.	Ether	Insoluble	Soluble
3.	Methanol	Insoluble	Insoluble
4.	Salt solution	Insoluble	Soluble
5.	Dilute hydrochloric acid	Soluble	Soluble
6.	Dilute sodium hydroxide	Soluble	Soluble

UV-Visible spectral characterization:

In the colorimetric estimation, stock solutions of glutamic acid and aspartic acid was prepared in water. From this solution, aliquots of 1mL, 2mL,….were pipetted out into a series of 10 mL volumetric flask to get the required concentration range. A 1 mL of sample solution was taken and mixed with 1 mL, 0.5% w/v solution of ninhydrin in methanol. Then 1 mL of pyridine heated on a water bath for 15 minutes. Cooled and added 0.2 mL of copper sulphate solution and diluted to 10 mL with methanol. The absorbance was measured at 500 nm, concentration of respective amino acids was calculated and results of the study are given in Table 2.

Table 2: Standard curve data for estimation of glutamic acid and aspartic acid at 500nm.

S. N.	Concentration (in µg/mL)	Absorbance at 500nm	
		Glutamic acid	Aspartic acid
1.	30	0.018	0.019
2.	36	0.021	0.021
3.	42	0.028	0.024
4.	48	0.031	0.030
5.	54	0.042	0.035
6.	60	0.047	0.040
7.	66	0.052	0.046

Infra red spectral characterization:

IR spectra were recorded on Shimadzu 8400S and Perkin Elmer RX1 FTIR spectrophotometer (Shimadzu Corporation, Japan) using KBr discs and the values are expressed in cm^{-1}.

Synthesis of polypeptides using modified Schotten-Baumann reaction

Synthesis of polyglutamic acid

Phthalic anhydride 5.9 g (0.04M) was added to glutamic acid 5.9 g (0.04M) and ground to a fine powder. This powder was heated on a water bath at 90°C for 15 minutes to get protected glutamic acid. Thionyl chloride 9.44 g (5.9 mL, 0.08) was added to 11.12 g (0.04M) of protected glutamic acid, in 100 mL of chloroform, and the contents were refluxed at 25-30°C under reduced pressure for 1 hour. The solvent and excess of thionyl chloride were distilled off under reduced pressure to get the protected glutamic acid chloride.

Potassium carbonate solution (10%) 50 mL was cooled on an ice-bath to 10°C. Glutamic acid 11.8 g (0.08M) was added in small portions with continuous stirring. Protected glutamic acid chloride 12.6 g (0.05M) was added to the alkaline amino acid solution in portions with constant stirring for 2 hours at 10 °C. The separated compound was washed with 0.5% cold sodium hydroxide solution and recrystallized. The procedure was repeated for several steps to get the protected polyglutamic acid.

Hydrazine hydrate (0.04M) was taken and cooled on an ice-bath to 15 °C. Protected polyglutamic acid was (0.04) added in small portions with continuous stirring for 2 hours. The separated compound was washed twice with a 5:1 ether and methanol solution, then with ether and dried under vacuum.

Synthesis of polyaspartic acid

Phthalic anhydride 5.9 g (0.04M) was added to glutamic acid 5.3 g (0.04M) and ground to a fine powder. This powder was heated on a water bath at 90°C for 15 minutes to get protected aspartic acid. Thionyl chloride 9.44 g (5.9 mL, 0.08) was added to 10.56 g (0.04M) of protected aspartic acid, in 100 mL of chloroform, and the contents were refluxed at 25-30°C under reduced pressure for 1 hour. The solvent and excess of thionyl chloride were distilled off under reduced pressure to get the protected aspartic acid chloride.

Potassium carbonate solution (10%) 50 mL was cooled on an ice-bath to 10°C. Aspartic acid 11.8 g (0.08M) was added in small portions with continuous stirring. Protected aspartic acid chloride 12.6 g (0.05M) was added to the alkaline amino acid solution in portions with constant stirring for 2 hours at 10 °C. The

separated compound was washed with 0.5% cold sodium hydroxide solution and recrystallized. The procedure was repeated for several steps to get the protected polyaspartic acid.

Hydrazine hydrate (0.04M) was taken and cooled on an ice-bath to 15 °C. Protected polyaspartic acid was (0.04) added in small portions with continuous stirring for 2 hours. The separated compound was washed twice with a 5:1 ether and methanol solution, then with ether and dried under vacuum.

Physicochemical characterization of synthesized polypeptides

The approximate molecular weight of the polypeptides was determined by Sorensen's method. A protein or amino acid cannot be estimated by direct titration with standard alkali solution owing to the opposing effects of the basic and acidic groups. If, however, the amino acids are first treated directly with neutral formaldehyde solution, it can be treated directly with standard sodium hydroxide solution. The polypeptides were completely hydrolyzed using excess alcoholic potassium hydroxide and back titrated with standard hydrochloric acid. The approximate equivalent weight of the substances were calculated using the quantity of potassium hydroxide consumed. [12]

Physical characterization and solubility studies:

Physical characterization and solubility studies data of the synthesized polypeptides are given in Table 3.

IR spectra:

IR spectra were recorded on Shimadzu 8400S and Perkin Elmer RX1 FTIR spectrophotometer (Shimadzu Corporation, Japan) using KBr discs and the values are expressed in cm^{-1}, as given in Figure 1.

Gel electrophoresis:

The gel electrophoresis (SDS-PAGE) method was also used for the determination of molecular weight. The photographs are depicted in Figure 2.

Matrix-assisted laser desorption ionization- Time of flight (MALDI-TOF):

The low volatility of polypeptides makes mass spectrometry useless for their investigation. This has been circumvented by the introduction of techniques for effectively dispersing proteins and other macromolecules into gas phase and the method is called matrix-assisted laser desorption ionization (MALDI) and electrospray spectrometry. In this technique, the polypeptide ions are generated and then accelerated through an electrical field. They travel through the flight tube, with the smallest travelling fastest and arriving at the detector first. Thus, the time of flight (TOF) in the electric field is a measure of the mass, or the mass/charge ratio. MALDI-TOF is indeed an accurate means of determining protein mass.

Biodegradation studies of synthesized polypeptides

Polymer degradation can be classified into photo and photo-oxidative, thermo and thermo-oxidative, mechanochemical, ozone-induced, radiolytic, ionic and biodegradation. Biodegradation of polymers is the classification of greatest importance for biomedical, pharmaceutical and environmental applications [13]. Biodegradation is the conversion of materials into less complex intermediates or end products by solubilization, simple hydrolysis or the action of biologically formed entities which can be enzymes and other products of the organism [14, 15]. Biodegradation occurs through four different mechanisms: Solubilization, charge formation followed by dissolution, hydrolysis and enzyme catalyzed degradation. Since, it is quite often difficult to distinguish the various mechanisms of degradation occurring *in vivo*; the term biodegradation is used, unless distinctions among them are absolutely necessary [16]. Biodegradation of polymers is expected to undergo four stages: Hydration, strength loss, loss of mass integrity and loss of mass. The hydration of polymers depends on the hydrophilicity of the polymer and results from disruption of secondary and tertiary structures stabilized by van der Waals forces and hydrogen bonds. During and after hydration, the polymer chains become water soluble and may cleave by chemical or enzyme catalyzed hydrolysis. Hydrolysis of the polymer backbone is most desirable since it will produce low molecular weight by-products and it is well known that the natural polymers such as proteins and polysaccharides undergo degradation by hydrolysis [17].

In-vitro hydrolysis of synthesized polypeptides

In vitro hydrolysis studies in simulated gastric fluid and simulated intestinal fluid truly mimics the *in vivo* milieu outside the body providing an artificial environment to drugs administered through oral route. Therefore, this study could be indicative in assessing the biodegradation of the synthesized polypeptides.

Polypeptides, 5mg each were separately and accurately weighed, dissolved in minimum quantity of ethanol and transferred into flasks, containing 100 mL of simulated gastric fluid (SGF), kept on a mechanical shaker at 37 °C. Samples of 2 mL were removed at half an hour intervals from each flask, diluted to 10 mL in volumetric flask and the absorbance measured against a SGF blank, using a Thermospectronic Genysis 10 spectrophotometer. The same procedure was followed for the hydrolysis of the polypeptide in simulated intestinal fluid (SIF).

The polypeptides, PGA and PAA were subjected to hydrolysis for a period of four hours. The aliquots removed did not give any absorbance at 500 nm. Thus, it can be concluded that after four hours, the polypeptides were not hydrolyzed to single amino acids, which would have otherwise shown absorbance at 500 nm. However, the hydrolysis of polypeptides to smaller peptides is not ruled out.

***In-vitro* screening of synthesized polypeptides for antibacterial activity**

The antibacterial activity [18-23] study of the polypeptides was performed in order to ensure: The potency of the polypeptide in solution and; the sensitivity of selected microbes to the known concentration of polypeptides. Different methods [24-27] are in practice, each with its own limitations and advantages. These include agar diffusion (cup, disc and cylinder) method, serial dilution method, turbidimetric method etc. with the first two being used most often. In the present study, agar diffusion with disc method was followed, selected on the grounds of convenience, sensitivity and practice.

Preparation of culture media

The formula used for preparing the culture media was as follows: Beef extract (1g), yeast extract (2g), peptone (5g), sodium chloride (5g), distilled water up to (1000 mL).

Accurately weighed quantities were dissolved in the prescribed quantity of fresh distilled water. The pH was adjusted to 7.2 to 7.4 by addition of 0.1 N sodium hydroxide solution. The media was then sterilized by autoclaving at 121°C, 15 pounds psi pressure for 2 hours [28].

A 2% w/v agar media was prepared by using the following formula: Beef extract (1g), yeast extract (2g), peptone (5g), sodium chloride (5g), agar (20g), distilled water up to (1000 mL).

Accurately weighed quantities of all the ingredients, except agar, were dissolved in a small quantity of distilled water by heating on a water bath. The solution was filtered through a muslin cloth. The pH of the solution was adjusted to 7.2 to 7.4 by adding 0.1 N sodium hydroxide solution. Then, weighed quantity of agar was added and the volume made up with distilled water. The media was then sterilized by autoclaving at121°C, 15 pounds psi pressure for 2 hours [29].

Proper diffusion of the compound through the media is a necessity in determining the antibacterial activity. Thus, water solubility of compounds is desirable [30]. However, since the synthesized compounds were sparingly soluble in water, 10% dimethyl formamide (DMF) was used. This concentration of DMF was considered to possess no antibacterial activity by itself [31].

The selection of micro-organism were such as to include both gram-negative and gram-positive bacteria. The strains selected were *Proteus morganii* and *Staphylococcus aureus.*

Preparation of test samples

Polypeptide samples (10 mg each) was dissolved in DMF and aliquots with 0.1 mL and 0.5 mL were withdrawn and diluted to 10 mL with sterilized distilled water to get solutions of 10 µg/mL and 50 µg/mL concentration of each polypeptide.

Preparation of inoculums

The inoculums were prepared by transfering a loopeful of the corresponding micro-organism from the stock culture into the sterile broth and incubating. A two days old young culture of the concentration of $3x10^{-6}$/μL was used.

Determination of anti-bacterial activity

The sterilized nutrient agar was poured into the petri dishes aseptically. A swab of absorbent cotton was soaked in the inoculums and gently applied on the surface of the cooled agar plates to get a uniform distribution of the cultures. Sterile filter paper discs (Whatman No. 1) of 8 mm diameter were soaked in the test solution. Each plate was labeled according to the micro-organism and test solution used. A filter paper disc from each solution was carefully kept on the surface of the respective agar plate. The plates were incubated at 37°C for 72 hours. The diameter of the zones of inhibition was then recorded.

Results and discussion

The bioactive polypeptides were synthesized by phthalimide protection, Schotten-Baumann reaction and hydrazine hydrate deprotection method. The physicochemical properties of the polypeptides were investigated and are given in Table 3.

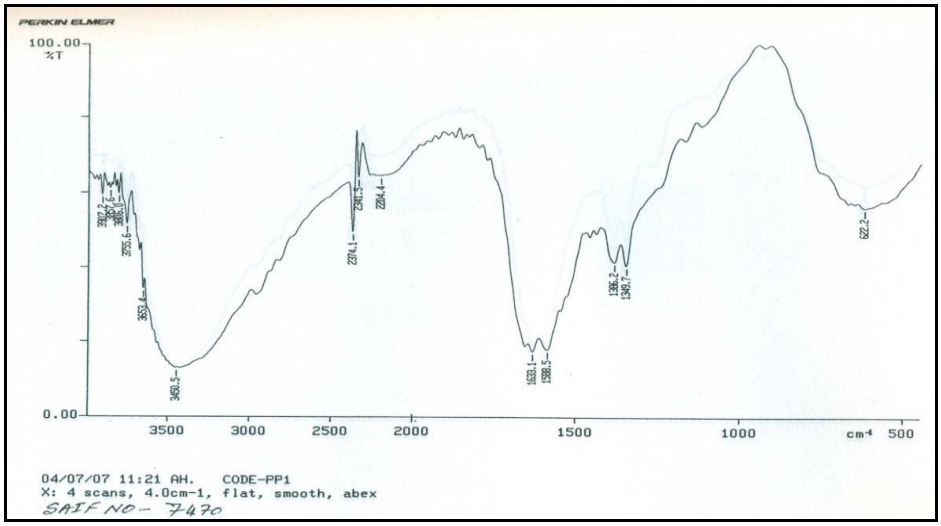
Table 3: Physical characterization and solubility studies data of the synthesized polypeptides.

S. N.	Physicochemical parameters		Poly-glutamic acid (PGA)	Poly-aspartic acid (PPA)
1.	Colour		Off white	Creamish
2.	Odour		Very slight	Very slight
3.	Melting Range (°C)		135-142	122-128
4.	Solubility (in solvents)	Water	+	+
		Methanol	+++	+++
		Acetone	-	-
		Ethylene glycol	+	+
		Ether	-	-
		Benzene	-	-
		Chloroform	+	+
		Pyridine	+	+
		DMF	+++	+++

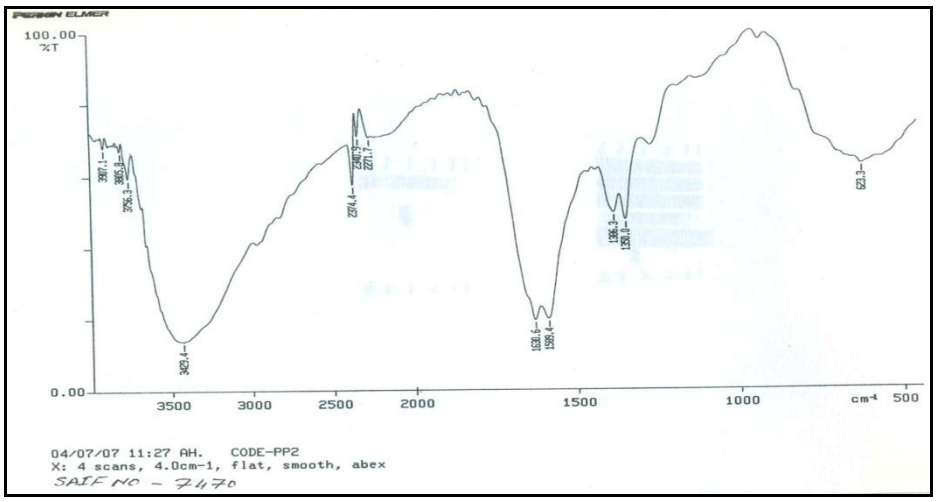
+++: Completely soluble; +: Sparingly soluble; -: Insoluble.

The organoleptic properties of the polypeptides were quite a contrast from the parent amino acids, and so were the melting point and solubility profiles. The solubility of PGA and PAA at 37°C, were in the order of: Methanol = Ethanol = DMF > Water = Chloroform = Pyridine > Ethylene glycol > Acetone = Ether = Benzene. This reflects hydrophobicity, due to the functional groups, and hydrophobicity/ lipophilicity, due to the chain, thus indicating the mixed or amphiphilic solubility behavior of the compounds.

The IR spectral studies of the synthesized polypeptides were conducted. The various peaks such as 3450-3000 for N-H stretching, 1750-1600 for C=O stretching, 1550-1500 for N-H bending, 1250-1200 for C-N vibration, 650 for out of plane N-H wagging confirmed the presence of amide/ peptide bond.



(1a)



(1b)

Figure 1: IR Spectra of- (1a) Poly-glutamic acid (1b) Poly-aspartic acid.

The molecular weight of the synthesized polypeptides were determined by Sorenson's method and gel electrophoresis (SDS-PAGE) method, thereby indicating macromolecules of molecular weight 48,000 and 66,000 approximately for PGA and PAA, respectively. The latest technique of molecular weight determination, MALDI-TOF further confirmed the molecular weights.

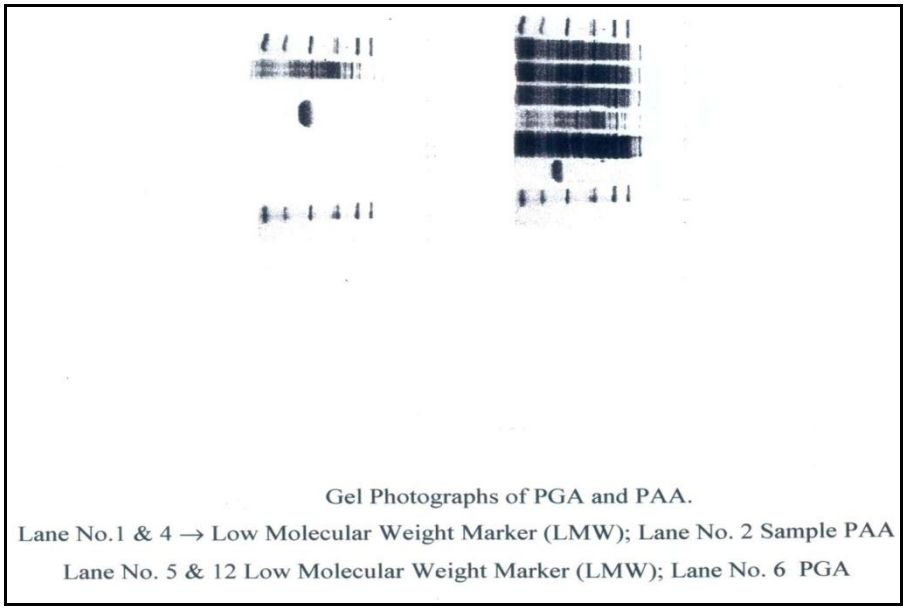


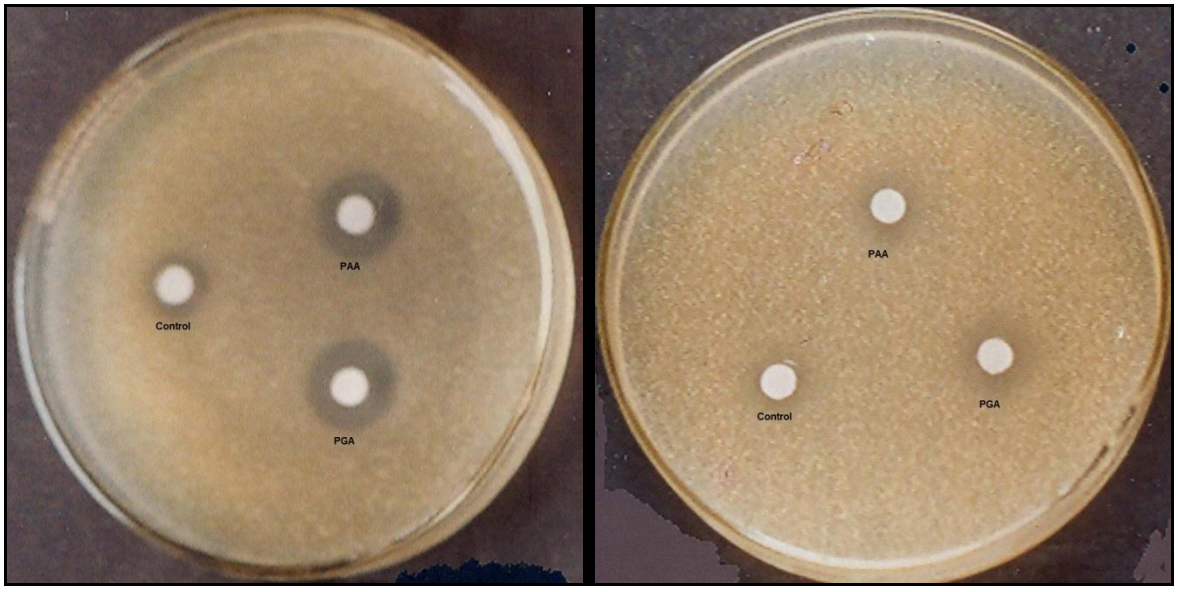
Figure 2: Gel electrophoresis photographs of PGA and PAA.

Acidic and alkaline hydrolysis of the polypeptides revealed that after four hours, no single unit of amino acid was formed. This indicated that the polypeptides should first degrade to larger units and then to smaller ones, thus indicating their stability and should provide a time lag in the drug delivery.

The antibacterial activity studies were conducted against Gram negative as well as Gram positive microorganisms at different concentrations. The antibacterial activities of the synthesized polypeptides PGA and PAA were found significant at higher doses when compared to control, but lesser than the standard drug ciprofloxacin. The antibacterial activity of PGA was found to be better than that of PAA. This could be due to better cell wall interaction of the compound. The results of antibacterial activity of synthesized PGA and PAA were recorded and are represented in Table 4 and Figure 3.

Table 4: Data showing antibacterial activity of synthesized polypeptides.

S. N.	Polypeptide	Concentration (µg/mL)	Diameter of zone of inhibition (mm)	
			Proteus morganii	***Staphylococcus aureus***
1	Poly glutamic acid (PGA)	10	11	10
		50	13	13
2	Poly aspartic acid (PAA)	10	10	10
		50	11	10
3	Control (DMF)	10%	00	00
4	Standard	Ciprofloxacin	16	14



(4a) **(4b)**

Figure 3: Photograph showing zone of inhibition for antibacterial activity of PGA and PAA against (4a) *Proteus morganii*; (4b) *Staphylococcus aureus*.

The above results are indicative that the polyanionic polypeptides can exhibit biological activity by themselves, i.e. they can act as bioactive polymers. Also, such polypeptides shall be degradable in the physiological system by various specific and non specific proteolytic enzymes and even the smallest unit, the amino acid, shall be non toxic. This shall lead to less metabolic burden on the vital organs. Thus, the use of polyanionic polypeptides for their biological activity or as drug-carrier macromolecules could be a promising concept in drug development.

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References

1. Park, K., Shalaby, W.S.W., Park, H., Biodegradable hydrogels for drug delivery, Technomic Publishing Company, Pennsylvania, 1993, 237.
2. Ottenbrite, R.M., Takemoto, K. and Miyata, M., Biomedical polymers and polycarboxyllic acid polymer drugs in functional monomers and polymers, K. Takemoto, Y. Inaki, and R.M. Ottenbrite, (Ed)., Marcel Dekker Inc., N.Y., 1987, 423-459.
3. Klotz, I.M., Enzyme models: Synthetic polymers, in enzyme mechanisms, M.I., Page and Williams, (Ed), The Royal Society of Chemistry, London, 1987.
4. Ottenbrite, R.M., Anionic polymeric drugs: Structure and biological activities of some anionic polymers, John Wiley and Sons, New York, 1980, 21.
5. Ottenbrite, R.M., Takatsuka, R., Polymer systems for biomedical applications, J. Bioactive and Compatible Polymers, 1986, 1, 46.
6. Himaja Malipeddi Patel Samir and Vinodhini. V, Synthesis and biological evaluation of cyclo[(N-Me, O-Me) Tyr-LeuAla-Gly-Pro] a pseudostellarin-A analog, International Journal of PharmTech Research, 2016, 9(6), 250-256.
7. Himaja, M., Patel S., Das P., Synthesis of cyclo[tyrosyl-(N-Me)leucinyl-prolyl- threonyl(nitro) arginine]: A potent anthelmintic agent against *Eudrillus eugeniae*, International Journal of ChemTech Research, 2016, 9(6), 316-321.

8. Cleas, P., Billiau A., De Clerq, E., Desmyter, J., Shonne, E., Vanderhaeghe, H., and Desomer, P., J. Virol., 1970, 5, 313.
9. Boddy, A., Aarons, L., Pharmacokinetic and pharmacodynamic aspects of site specific drug delivery, Advanced Drug Delivery Reviews, 1989, 3(2), 155-266.
10. Goddard, P., Therapeutic proteins- a pharmaceutical perspective, Advanced Drug Delivery Reviews, 1991, 6(2), 103-233.
11. Horton, V.L., Blegen, P.E., Barrows, T.H., Quarfoth, G.L., Gibson, S.J., Jonson, J.D., McQuinn, R.L., Progress in biomedical polymers, Gebelein, C.G., Dunn, R.L. (Ed), Plenum Press, New York, 1990, 263.
12. Mann, F.G., Saunders, B.C., Practical organic chemistry, Orient Longman, New Delhi, 1999, 463-464.
13. Grassie, N., Scott, G., Polymer degradation and stabilization, Cambridge University Press, New York, 1985.
14. Holland, S.J., Tighe, B.J. Biodegradable polymers, In Adv. Pharmaceutical Sci., D. Ganderton and T. Jones (Ed), Academic Press, New York, 1992, 101-164.
15. Vert, M., Bioresorbable polymers for temporary therapeutic applications, Die Angewandte Macromolekulare Chemie, 1989, 155-168.
16. Gilding, D.K., Biodegradable polymers, Biocompatibility of clinical implant materials, D.F., Williams, (Ed), CRC Press, Boca Raton, 1981.
17. Schechter, K.K., Berger, A., The size of the active site in proteases. I. Papain, Biochem. Biophys. Res. Commun., 1967, 27(2), 157-162.
18. Patil, R. B., Sawant, S. D., Synthesis, characterization, molecular docking and evaluation of antimicrobial activity of some 3-heteroaryl substituted chromen-2-one derivatives, International Journal of PharmTech Research, 2014-15, 7(3), 471-480.
19. Malipeddi, H. and Das, M., Synthesis, anhelmintic, antimicrobial and anti-inflammatory activities of axinastatin-3, International Journal of ChemTech Research, 2015, 8(10), 90-95.
20. Thakur, A., Gupta, P. R. S., Pathak, P., Kumar, A. Design, synthesis, SAR, docking and antibacterial evaluation: Aliphatic amide bridged 4-aminoquinoline clubbed 1,2,4- triazole derivatives, International Journal of ChemTech Research, 2016, 9(3), 575-588.
21. Sangdee K., Pimta J., Seephonkai, P, Sangdee A, Antibacterial activity, time-kill profile and morphological effects of Streptomyces sp. SRF1 extracts against the foodborne pathogen *Bacillus cereus*, International Journal of ChemTech Research, 2016, 9(6), 709-717.
22. Jays, J. Kumar, A., Venketaramana, C. H. S., Suma, B. V., Madhavan, V., Synthesis and biological evaluation of some new 2-(2-Methyl-5-nitro-1H-imidazol-1-yl)-N'-[(3Z)-2-oxo-1, 2-dihydro-3H-indol-3-ylidene] acetohydrazide derivatives, International Journal of ChemTech Research, 2011, 3(2), 772-777.
23. Tiwari, B., Pratapwar, A. S., Tapas, A. R., Butle, S. R., Vatkar, B. S., Synthesis and antimicrobial activity of some chalcone derivatives, International Journal of ChemTech Research, 2010, 2 (1), 499-503.
24. Sharma, K.K., Sangraula, H., Mediratta, P.K., Indian Journal of Pharmacology, 2002, 34, 390-396.
25. Reynolds, J.E.F. The extra pharmacopoeia, 31st Ed., Royal Pharmaceutical Society of Great Britain, London, 2002, 207-210.
26. Gennaro, A.R., Remington: The Science and practice of pharmacy, 20th Ed., Vol-I, Lippincott Williams and Wilkins, Philadelphia, 2000, 540-551.
27. Fairbrother, M., A textbook of bacteriology, William Heinemann Medical Books Ltd., London, 1960, 36.
28. Rawlins, E.A., Bentley's textbook of pharmaceutics, 8th Ed., Balliere Tindall, London, 2001, 563-573.
29. Pelczar, M.J., Chan, E.C.S., Krieg, N.R., Microbiology, 5th Ed., Tata McGraw-Hill, New Delhi, 2002, 99-114.
30. Hugo, W.B., Russell, A.D., Pharmaceutical Microbiology, 6th Ed., Blackwell Scientific, Oxford, 2003, 439-452.
31. Tortora, G.J., Funke, B.R., Case, C.L., Microbiology-An Introduction, 8th Edition, Pearson Education, Singapore, 2004, 155-182.

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