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Synthesis, Physicochemical Properties and Biological Evaluation of Some Peptide Candidates by Use of Liquid Phase Method as Potential Antimicrobial and Surface Active Agents

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ABSTRACT
In order to obtain peptide based surface active agents with antimicrobial activity, a series of polyethylene glycol bounded peptide chains were synthesized (I-VI). The investigated peptide chains contain aliphatic and aromatic amino acids in different sequences. These compounds were prepared by use of liquid phase method loaded on polyethylene glycol with molecular weight 3000 Daltons as a polymeric support. The produced target peptides were characterized by means of their amino acid analysis, HPLC and UV spectroscopy. The physicochemical, surface active properties measurements and *in vitro* antimicrobial activity of the synthesized peptide chains were evaluated. The synthesized peptide chains were found to have promising surface properties beside their antimicrobial activity.

Key words: Peptide, physicochemical properties, surfactants, antimicrobial, liquid phase method, biodegradability

INTRODUCTION

In recent years many Antimicrobial Peptides (AMPs) were synthesized with respect to their possible application as chemotherapeutic agents (Carmona-Ribeiro and de Melo Carrasco, 2014; Naglah *et al*., 2014). N^{α}-lauroyl arginine and dipeptide were found to exhibit good surfactant properties and antimicrobial activity (Infante *et al*., 1989). This stimulates many studies aim to investigate the surfactant properties of amino acids and their derivatives. N-(2-hydroxyethyl)-N-(2-hydroxy alkyl)-β-alanine and their oxythylated derivatives were studied in blends with convention anionic surfactants. It was indicated that these systems have excellent detergency and other active surface properties (Takai *et al*., 1980). It has also been established that derivatives of amino acids containing the 2-perfluoroalkyl-2-hydroxyethyl group considered a good amphoteric surfactants with different application (Nasreddine *et al*., 1993). Amphoteric surfactants that include amino acids found to have spacious application like shampoos, cosmetics, emulsion, paints, textile industry and corrosion inhibition (Eissa, 2006). In view of these observations, some model peptide chains bounded to polyethylene glycol according to liquid phase peptide synthesis (Bayer and Mutter, 1972) were synthesized in order to evaluate their surface active properties and antimicrobial activity.

MATERIALS AND METHODS

Chemistry: The peptides were synthesized by use of liquid phase method with Boc-strategy and polyethylene glycol as soluble polymeric support. Solvents and chemicals were obtained from Sigma-Aldrich (USA) chemical company. The

used amino acids are of L-configuration (Ala: Alanine, Gly: Glycine and Tyr: Tyrosine). PEG with M. Wt. 3000 Daltons was obtained from Fluka (Switzerland) chemical company. All solvents were analytically pure and dried.

Coupling of PEG_{3000} with the first amino acid: The PEG_{3000} (1 mmol) was dissolved in 20 mL dichloromethane. Symmetrical anhydride of the N^a-tert. Boc-amino acid was synthesized in a reaction vessel by dropping (5 mmol) of 1,3-dicyclohexylcarbodiimide (DCC) to (10 mmol) N^a-tert. Boc-amino acid in dichloromethane, anhydride was filtered upon the polymer solution. Then 10 mL pyridine was added to the mixture, the volume was concentrated to 10 mL under vacuum, then the mixture was stirred for 12-24 h at room temperature. The produced compound was precipitated by addition of dry and cold diethyl ether drop by drop to the solution under stirring at 0°C; the product was filtered off, washed with diethyl ether. The produced compound was dissolved in small portion of suitable solvent and precipitated by addition of dry diethyl ether during cooling many times until pure product was obtained.

Removal of the (tert. Boc-group) N^a-Protected group: To deprotect PEG_{3000} amino acid-Boc, 2 mmol was dissolved in (20 mL) Trifluoroacetic acid/dichloromethane (1:1), stirring for 1/2 h at r. t, then the mixture was concentrated under vacuum; the product was dissolved in dichloromethane and precipitated by dropwise addition of dry diethyl ether during cooling with vigorous stirring till Kaiser test showed a positive result (Kaiser *et al*., 1970). The pure product was filtered off and dried under vacuum. The rest peptide sequences were synthesized according to the above steps.

Amino acid analysis: The amino acid composition of prepared peptide chains was determined by amino acid analysis using a LC3000 Eppendrof with an integrator system 1. Before that analysis samples were hydrolyzed in 6 N HCl in sealed and evacuated tubes at 110°C for 24 h.

Ultra Violet (UV) spectrometry: Ultra violet spectra were recorded on UV spectrophotometer, Shimadzu model 1601PC.

High Performance Liquid Chromatography (HPLC): Purification of prepared peptides using HPLC Chromatogram; Sykam S2000, 52110 Injector: Rheodyne 7125. UV Detector: Shimadzu, SPD, GAV. Detection at 220 nm, Integrator: Sykam, C-RGA Chromatopac. Column: Nucleosil 120 C18, 25 cm×4.6 mm. Solvent: A) 0.1% TFA in H_2O, B) 0.1% TFA in acetonitrile. Gradient: 0-50% B in 50 min.

Biological study: All samples were dissolved in DMSO at 10 mg mL^{-1} concentration, in compare with different standard antibiotics are illustrated. The ability to inhibit the growth of Gram-positive and Gram-negative bacteria and yeast were observed using an overlay method (Williams *et al*., 1983). The bacteria were slanted on nutrient agar, yeast was slanted on Sabaroud's agar medium and the fungi was slanted and mentioned on the potato Dextrose Agar medium. The disk diffusion was used for antibacterial screening and agar method described by Moosdeen *et al*. (1988). Plates will be incubated under aerobic conditions at 37 and 28°C for 24 and 48 h for bacteria and fungi. Plates were examined for evidence of antimicrobial activities, represented by a zone inhibition of microorganism's growth around the paper disk and diameters of clear zones were expressed in millimeters (Cruickshank *et al*., 1975).

SURFACE ACTIVE PROPERTIES

Surface and interfacial tension: The method for evaluation of surface active properties are described in Du-Nouuy's interfacial tensiometer (Kruss, type 4851) was used taking distilled water at 25°C as 73.1 dyne cm^{-1} and the interfacial tension between medicinal paraffin oil and distilled water as 56.2 dyne cm^{-1}.

Wetting properties: The wetting power for the prepared peptides was evaluated by measuring the sinking time in seconds of a grey cotton skin in the surfactant solutions (0.5% by weight) at 28°C.

Foaming properties and emulsion stability: Foaming properties were tested according to Ross-Millis method (Ross and Miles, 1941). On other hand, the emulsion was prepared by adding 10 mL of a 20 mmol aqueous solution of the surfactants to 5 mL toluene at 40°C. The emulsifying function was identified by the time it took for an aqueous volume separating from emulsion layer to reach 9 mL counting from the instant of shaking was stopped.

Cloud point and stability to hydrolysis: The cloud point of the individual chains was determined as reported method (Wiel *et al*., 1963), as well in case of stability to hydrolysis, a mixture of 10 mL of the 10 mmol surfactants and 10 mL of 2 N sulphuric acid was placed in a thermostat 40°C. The time takes for a sample solution to be clouded as the results of hydrolysis shows the stability of the surfactant to hydrolysis (Amine *et al*., 2004).

Critical Micelle Concentration (CMC): The critical micelle concentration values for the prepared peptide chains were determined by the electrical conductivity method (Takeshi, 1970).

Biodegradability: Samples taken every day were filtered via filter paper before measuring the surface tension. Surface

tension determinations were made periodically on each sample during degradation test. Biodegradation percent (D) for each sample was calculated using the following equation:

$$D = \frac{\gamma_t - \gamma_0}{\gamma_{bt} - \gamma_0} \times 100$$

Where:

γ_t = Surface tension at time t
γ_0 = Surface tension at zero time
γ_{bt} = Surface tension of blank experiment at time t

RESULTS AND DISCUSSION

Chemistry: In attempt to investigate the surface properties of the polymer bounded peptides which possess antimicrobial activity, the following peptide chains were synthesized using LPPS (Scheme 1) and the chemical structure of peptide chain No. I was shown in Fig. 1.

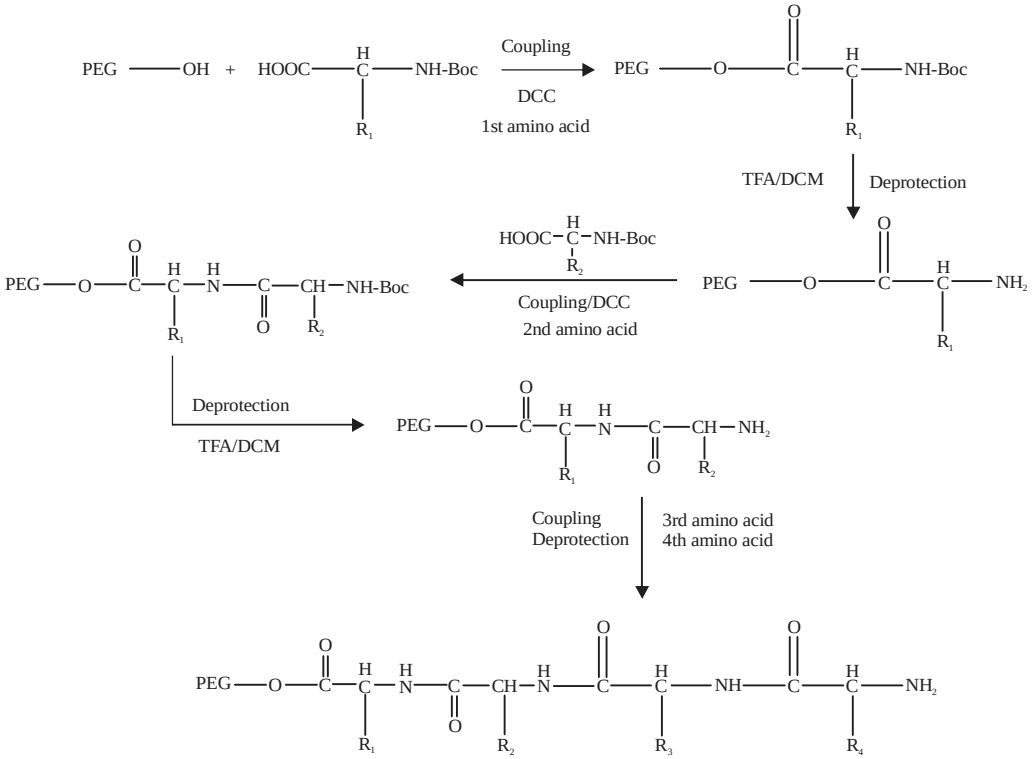
Peptide No.	Peptide sequence
I	PEG-Gly-Gly-Tyr-Ala-NH_2
II	PEG-Gly-Tyr-Ala-Gly-NH_2
III	PEG-Gly-Gly-Ala-Ala-NH_2
IV	PEG-Gly-Gly-Tyr-Tyr-NH_2
V	PEG-Gly-Tyr-Tyr-Ala-NH_2
VI	PEG-Gly-Gly-Ala-Tyr-NH_2

These peptides are covalently bounded to monofunctional polyethylene glycol of molecular weight 3000 Daltons which exerts a strong solubilizing effect on the attached peptide chains in all solvents. The properties of PEG allow its penetration during the cell membrane, so the biological evaluation could be implemented on PEG-bounded peptides (Abdel Rahman and Hattaba, 1988).

The Boc-group was worked as N-terminal protecting group and the first amino acid was covalently bounded to PEG. Coupling reactions were monitored by ninhydrin and Kaiser test. The Boc-group was deprotected by dissolving the PEG-peptides in the mixture of Trifluoroacetic acid/dichloromethane (1:1) with stirring for 1/2 h at r. t. The crude PEG-peptides were collected by dissolving in distilled water, lyophilized, subjected to HPLC and identified by spectral analysis.

Amino acid analysis: Amino acid analysis pointed to the methodology used to determine the amino acid composition of peptides. The results of the amino acid analyses are very close to the theoretical values expected for the same sequences (Table 1). These data confirm the presence of all the amino acids in the peptide backbone during chemical preparation.

Ultra Violet (UV) spectroscopy: The synthesized PEG-peptide chains were confirmed by the application of ultraviolet measurements and the results were illustrated in Table 2.



Scheme 1: Synthetic routes for the peptide chains (I-VI)

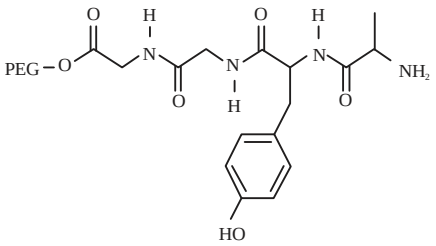


Fig. 1: Chemical structure of peptide chain No. I

Table 1: Amino acid analysis for the synthesized peptide chains

	Amino acids		
Peptide No.	Ala	Tyr	Gly
I			
Calculated	1	1	2
Found	1	0.8	1.96
II			
Calculated	1	1	2
Found	0.98	0.86	2.1
III			
Calculated	2	-	2
Found	1.92	-	1.97
IV			
Calculated	-	2	2
Found	-	1.86	1.99
V			
Calculated	1	2	1
Found	1	1.67	0.99
VI			
Calculated	1	1	2
Found	0.96	0.89	2

Ala: Alanine, Tyr: Tyrosine and Gly: Glycine

Table 2: UV absorption bands of the PEG-Peptides

Peptide No.	W. L.	Abs.
I	230	1.181
II	229	1.322
III	228	No beak
IV	231	2.262
V	228	2.334
VI	228	1.486

Abs: Absorbance

From the above results, it was observed that the compound III showed no observed beak. Otherwise, the compounds I, II, IV, V and VI showed evident absorbance bands at λ_{max} 230, 229, 231, 228 and 228 nm, respectively, which may be attributed to the presence of chromophoric groups (Tyrosine) in the backbone structure of the synthesized peptides.

Antimicrobial activity: The results of antimicrobial activity of the synthesized peptides were summarized in Table 3 and were assayed against Gram-positive, Gram-negative organisms and yeast. The investigated peptide chains II and VI showed high activity against *C. albicans* compared to other chains. Peptide chains I and V have moderate activity against *B. subtilis*. All tested chains showed no activity against *E. coli* and *S. aureus*.

Physicochemical and surface active properties measurements: The present study represents new synthesized polymer bounded peptide chains with antimicrobial activity and promising surface properties suitable for wide area of applications. The surface tension of the under investigated chains were measured at room temperature by Du Nouy method (Du Nouy, 1919). Results were illustrated in Table 4 and showed the tested peptide chains produced a reduction of surface tension ranged from 32.3-37.5 dynes cm^{-1}. Chain V found to be the most effective one while chain III was the least active in this respect. Critical Micelle Concentration (CMC) values of the synthesized peptides were determined by applying the electrical conductivity methods. The values of CMC measurements which ranged from 6.2×10^{-2}-3.2×10^{-5} mmole L^{-1}. Table 4 indicated that the tested compounds posses good surface properties.

The performances of the tested substances were given in terms of foaming and wetting power, emulsion, cloud point and stability to hydrolysis. Results in Table 4 showed that the investigated peptides are low to moderate foaming. These low

Table 3: Antimicrobial activity of the synthesized compounds at 10 mg mL^{-1}

	Bacteria			
	Gram +Ve		Gram -Ve	Yeast Unicellular
Peptide No.	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
I	12.13±0.1768	00.00±0.0000	00.00±0.0000	11.12±0.1465
II	00.00±0.0000	00.00±0.0000	00.00±0.0000	30.15±0.1566
III	00.00±0.0000	00.00±0.0000	00.00±0.0000	15.10±0.1414
IV	00.00±0.0000	00.00±0.0000	00.00±0.0000	14.13±0.1213
V	13.10±0.1414	00.00±0.0000	00.00±0.0000	13.11±0.1311
VI	00.00±0.0000	00.00±0.0000	00.00±0.0000	32.14±0.1523
S = 10 µg	14.00±0.0000	00.00±0.0000	12.00±0.0000	00.00±0.0000
TE = 30 µg	18.00±0.0000	00.00±0.0000	23.50±2.1213	00.00±0.0000
N = 30 µg	00.00±0.0000	00.00±0.0000	00.00±0.0000	16.00± 1.4142

Table 4: Physicochemical and surface properties of the synthesized peptide chains

Compound No.	Surface tension (dynes cm^{-1} 0.1 m L^{-1})	Interfacial tension (dynes cm^{-1} 0.1 m L^{-1})	Emulsion stability (sec)	Cloud point (°C)	Foam height (mm)	Wetting time (sec)	Stability to hydrolysis (min:sec)	CMC (mmol L^{-1})
I	34.8	7.3	215	86	66	130	22: 26	5.7×10^{-4}
II	32.9	6.9	340	87	78	106	18: 23	1.1×10^{-4}
III	37.5	8.9	198	94	63	137	30: 46	3.2×10^{-5}
IV	33.6	7.4	302	76	102	112	15: 36	1.3×10^{-2}
V	32.3	6.5	282	73	112	114	16: 02	6.2×10^{-2}
VI	34.7	7.7	217	80	76	120	32: 13	6.0×10^{-4}

CMC: Critical micelle concentration

Table 5: Biodegradability of the synthesized peptide chains

	Days						
Peptide No.	1st	2nd	3rd	4th	5th	6th	7th
I	47	54	66	82	91	98	--
II	45	56	65	72	83	91	99
III	52	57	72	79	83	97	--
IV	34	50	61	68	75	89	97
V	38	44	58	62	72	85	96
VI	39	53	61	70	78	88	90

foaming effects may be attributed to the presence of many hydrophilic groups which cause a considerable increase in the area per molecule and produce less cohesive forces at the surface. Examination of wetting properties of the peptide chains showed that they posses a potent wetting inducing efficiency. The dilute solution of all peptide chains could wet the cotton skeins in periods ranging from 106-137 sec. In this respect, these compounds may be potentially useful in variety of applications where wetting is desired e.g., dyeing processing, paintings, cosmetics and many other operations (Eissa, 2006). The cloud points of the individual chain were listed in Table 4. The results indicated that the values of cloud point increases by increasing the number of hydrophilic groups and decreases by the presence of aromatic rings. So, compounds IV and V showed low cloud points since each of them contains two Tyr residues. Stability to acid hydrolysis was also tested and the chains showed good stability towards acid hydrolysis. Emulsifying stability of the peptide chains was estimated and results indicated that the all peptide chains exhibit to adequate emulsification stability specially compounds II, IV and V. Member of this series could produces oil/water emulsion of considerable stability. This shows that the peptide chains under investigation could be useful in textile processing and dye bathes.

Biodegradability was also tested using Die-away test in the river water (Wylie *et al*., 1982) and the results were given in Table 5. All compounds showed high rate of the degradation reach 99% during 7 days. The results of biodegradation reflect the fact that the biodegradability decreases by increasing the branching and aromatic rings.

The above mentioned results of physicochemical and surface active properties indicated that the polyethylene glycol bounded peptides may be potentially useful as antimicrobial surface active agent posses pronounced surface activities.

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