Chinese Chemical Letters 26 (2015) 479–484

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Original article

Kinetic study of all-or-none hemolysis induced by cationic amphiphilic polymethacrylates with antimicrobial activity

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ARTICLE INFO

Article history:
Received 13 November 2014
Received in revised form 15 January 2015
Accepted 16 January 2015
Available online 7 February 2015

Keywords:
Hemolysis
Antimicrobial polymer
Amphiphilic polymethacrylate
Kinetic analysis
Membrane disruption

ABSTRACT

To gain an understanding of the toxicity of antimicrobial polymers to human cells, their hemolytic action was investigated using human red blood cells (RBCs). We examined the hemolysis induced by cationic amphiphilic methacrylate random copolymers, which have amino ethyl sidechains as cationic units and either butyl or methyl methacrylate as hydrophobic units. The polymer with 30 mol% butyl sidechains ($\mathbf{B_{30}}$) displayed higher hemolytic toxicity than the polymer with 59 mol% methyl sidechains ($\mathbf{M_{59}}$). $\mathbf{B_{30}}$ also induced faster release of hemoglobin from RBCs than $\mathbf{M_{59}}$. A new theoretical model is proposed based on two consecutive steps to form active polymer species on the RBC membranes, which are associated to RBC lysis. This model takes the all-or-none release of hemoglobin by the rupture of RBCs into account, providing new insight into the polymer-induced hemolysis regarding how individual or collective cells respond to the polymers.

1. Introduction

Due to the emerging issue of drug-resistant bacterial infections, there is a compelling need for the development of new antimicrobials that are effective against drug-resistant microbes [1]. To that end, we have previously developed a series of antimicrobial polymers based on random methacrylate copolymers [2–4]. These cationic amphiphilic polymers are designed to mimic the structural features and membrane lysis function of naturally occurring antimicrobial peptides. This class of polymers displayed a broad spectrum of activity [5], rapid bactericidal kinetics [6], and low propensity for resistance development in bacteria [6], providing a promising molecular platform to develop antimicrobial agents effective against drug-resistant bacteria.

Toward the implementation of these synthetic antimicrobial polymers to biomedical applications, it is important to improve their antimicrobial potency as well as reduce their toxicity to human cells. It would be necessary to increase our knowledge on the molecular mechanism of toxic activity of the polymers to elucidate the rational design of non-toxic antimicrobial polymers. Accordingly, this study focuses on the investigation of mechanism of polymer toxicity to human cells through a kinetic analysis of hemolysis of red blood cells (RBCs). Hemolytic activity has been used as an initial assessment of toxicity, reflecting physical damage to human cell membrane integrity [7,8]. Our previous study demonstrated that increasing polymer hydrophobicity increases the hemolytic activity of polymers. Hemolysis proceeds via partitioning of hydrophobic side chains into the RBC membranes, followed by membrane disruption [9]. The polymethacrylate derivatives induce the formation of nanosized pores (1.6–2.0 nm) in the membrane. The nanosized pore formation allows only influx of water and small solutes into the cells, but not efflux of large cytosolic proteins, resulting in osmotic imbalance between the cytosol and extracellular buffer solution, which contains only small salts. This causes lysis or complete rupture of RBCs (osmolysis), resulting in the release of entire cellular contents, we have also reported that this polymer-induced osmotic hemolysis is an all-or-none event, in which a fraction of RBCs are completely lysed at a given polymer concentration, while the remaining cells are still intact (Fig. 1B) [10]. The experimental data presented in Fig. 1C indicated that the percentage of disappeared RBCs after an addition of the polymer was proportional to the percent of hemoglobin release (the extent of hemolysis), suggesting an all-or-none response of RBCs in the polymer-induced hemolysis. This all-or-none hemolysis by the polymer is contrasted

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http://dx.doi.org/10.1016/j.cclet.2015.01.029

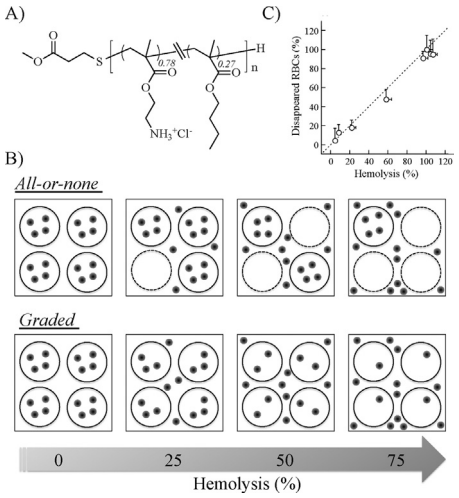

Fig. 1. All-or-none hemolysis induced by amphiphilic polymethacrylates [10]. (A) Chemical structure of the polymer. (B) Schematic presentation of all-or-none release and graded release of hemoglobin from RBCs. Filled circles correspond to hemoglobin. (C) Relationship between disappeared RBCs and the amount of released hemoglobin from RBCs (extent of hemolysis) induced by the polymer. Reprinted with permission Copyright 2011 American Chemical Society.

with a graded release mechanism proposed for some hemolytic peptides such as melittin [11] and γ-lysin [12], in which a potion of hemoglobin is leaked from all of the RBCs in a sample (Fig. 1B). Based on these previous findings on the all-or-none hemolysis, herein we report a new kinetic model to describe polymer-induced hemolysis. To the best of our knowledge, the all-or-none response in hemolysis seems not to be taken into account for the hemolysis models of peptides in literature. The kinetic analysis and associated new model would provide new insight into the molecular behaviors of polymer chains on the membranes such as polymer cooperativity as well as identify active species.

2. Experimental

All experimental procedures including materials, polymer synthesis, antimicrobial assay, hemolysis assay and kinetic measurement of hemolysis were described in the Supporting information.

3. Results and discussion

3.1. Polymer structure–activity relationships

We have synthesized two amphiphilic methacrylate random copolymers containing primary ammonium groups as cationic functionality and either butyl or methyl groups as hydrophobic side chains according to the previously reported procedure (Table 1, Fig. 2) [9]. The molecular weights of polymers are in the range of 2000–4000 g/mol, which mimic the small molecular size of natural antimicrobial peptides. The dansyl fluorescent dye at the polymer end is intended for measurement of binding affinity of polymers to lipid membranes [13]. The results will be reported elsewhere.

The copolymers **M_{59}** and **B_{30}** displayed potent antimicrobial activity with minimum inhibitory concentrations (MIC) values of 8 μg/mL (3.6 μmol/L) and 16 μg/mL (4.9 μmol/L), which are comparable to that of bee venom toxin melittin (MIC = 13 μg/mL or 4.6 μmol/L) and higher than that of natural antimicrobial peptide magainin-2 (MIC = 125 μg/mL or 50 μmol/L) under the same assay condition. The MIC values of the polymers reported in this study are in good agreement with those reported previously [9].

3.2. Dose–response hemolysis curves

To assess the toxicity of polymers to human cells, the ability of the polymers to lyse human RBCs was evaluated by monitoring release of hemoglobin in solution. In general, the fraction of hemoglobin release increased with increasing polymer concentration (Fig. 3). The HC_{50} values (Table 1), defined as the polymer concentration necessary to induce 50% hemoglobin release, were determined by fitting the Hill equation. The HC_{50} data indicate that the hemolytic activity of **B_{30}** (HC_{50} = 0.68 μmol/L) is comparable to melittin (HC_{50} = 0.71 μmol/L). The HC_{50} of **B_{30}** is more than 30 times lower than **M_{59}** (HC_{50} = 20 μmol/L), suggesting that more hydrophobic butyl groups confer greater hemolytic activity of polymers than methyl groups, in agreement with our previous report [9]. It has been also previously reported that magainin-2 displays little or no hemolytic activity under the same assay condition (HC_{50} > 100 μmol/L) [10]. The selectivity index defined as HC_{50}/MIC of **B_{30}** found to be 0.14, reflecting the non-selective activity of **B_{30}** against human cells and bacteria. In contrast, the **M_{59}** displayed higher selectivity index (HC_{50}/MIC = 5.6), indicating **M_{59}** is selectively active to bacteria over RBCs. These results suggest that the hemolytic activity and cell selectivity of polymers depends on their hydrophobic properties represented by the identity and their compositions of hydrophobic groups.

We have previously demonstrated that the polymer-induced hemolysis is an all-or-none event [10]. Since the hemolytic mechanism of our polymers differs from graded release, which has been proposed for some hemolytic peptides [11,12], a new kinetic model is required to accurately describe their mechanism of action. In the all-or-none hemolysis, the dose–response curves represent the *number* of RBCs that are lysed at a given time point and polymer concentration. The concentration dependence of

Table 1
Characterization and biological activities of amphiphilic methacrylate random copolymers.

Polymer or peptide	DP[a]	f_{HB}[b]	M.W.	MIC[c]		HC_{50}[d]		n[e]	HC_{50}/MIC
				μg/mL	μmol/L	μg/mL	μmol/L		
M_{59}	12	0.59	2200	8	3.6	44	20	1.7	5.6
B_{30}	14	0.30	3200	16	4.9	2.2	0.68	1.2	0.14
Magainin-2[f]	–	–	2500	125	50	>250	>100	–	>2
Melittin[f]	–	–	2800	13	4.6	2.0	0.71	N.R.[g]	0.15

[a] Degree of polymerization.
[b] Mole fraction of alkyl side chains.
[c] Minimum inhibitory concentration against *Escherichia coli*.
[d] Polymer concentration necessary for 50% lysis of human red blood cells.
[e] Hill coefficient obtained by the fitting analysis of dose–response curve.
[f] The MIC and HC_{50} were previously reported [10].
[g] Value was not reported.