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Circular Dichroism studies on the interactions of antimicrobial peptides with bacterial cells

SUBJECT AREAS:

PEPTIDES

BIOPHYSICAL CHEMISTRY

Received

11 September 2013

Accepted

17 February 2014

Published

12 March 2014

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Studying how antimicrobial peptides interact with bacterial cells is pivotal to understand their mechanism of action. In this paper we explored the use of Circular Dichroism to detect the secondary structure of two antimicrobial peptides, magainin 2 and cecropin A, with *E. coli* bacterial cells. The results of our studies allow us to gain two important information in the context of antimicrobial peptides- bacterial cells interactions: peptides fold mainly due to interaction with LPS, which is the main component of the Gram negative bacteria outer membrane and the time required for the folding on the bacterial cells depends on the peptide analyzed.

The emergence of bacterial strains resistant to common antibiotics has strongly encouraged studies on antimicrobial peptides (AMPs) from natural sources and on their mechanism of action^{1,2}. It has been demonstrated that antimicrobial peptides impair cell viability by mechanisms which likely depend on their sequence and structure³. Biophysical studies carried out using molecules which mimic the outer leaflet of bacterial cells, as lipid mixtures or purified lipopolysaccharides (LPS), led to hypothesize three different mechanisms by which peptides kill bacterial cells, namely the barrel stave, toroidal pore and carpet mechanism⁴⁻⁷. According to those mechanisms, antimicrobial peptides interact with bacterial outer membranes, perturb their integrity causing their disgregation. Nuclear Magnetic Resonance (NMR) and Circular Dichroism (CD) studies carried out in the presence of either detergents, as mimic of the bacterial outer leaflet, or LPS, which is the main component of the Gram negative bacteria outer membrane, allowed the determination of the three-dimensional and secondary structure of antimicrobial peptides in cell-like environments⁸⁻¹². A different perspective is actually offered by solid state NMR studies, which give interesting insights on the changes occurring to the membranes after interaction with the antimicrobial peptides, other than on the membrane bound structure of peptides¹³⁻¹⁵.

It is not clear yet how closely the results of these studies reproduce what really happens when antimicrobial peptides meet bacterial cells. Solid state NMR studies carried out with membranes of different composition demonstrated that the structure, dynamics and orientation of the peptides on the membrane is strictly related to the membrane composition¹⁶.

The envelope of bacteria is a very complex system¹⁷. In Gram negative bacteria it is an asymmetric bilayer having the inner leaflet composed of phospholipids, as phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, a peptidoglycan cell wall and the outer leaflet composed mainly of LPS and proteins as lipoproteins and beta barrel proteins as porins^{17,18}. On the outer membrane reside also some enzymes, for example the *E. coli* membrane contains a phospholipase (PldA), a protease (Omp T) and a LPS modifying enzyme (PagP). The LPS is absent on the Gram positive bacteria outer membrane, which possess instead a very thick peptidoglycan layer to which wall teichoic acids are covalently linked; lipoteichoic acids are deeply inserted into the peptidoglycan and are attached to the head group of membrane lipids. Membrane proteins are also embedded into the inner leaflet of Gram positive cell membrane.

This picture of the bacterial membrane envelopes suggests that the lipid mixtures or LPS employed in studies aimed at determining the structure assumed by antimicrobial peptides on bacterial cells or their mechanism of action are a simplified model system. In addition, while the outer leaflet of bacterial cell is a bilayer, purified LPS self-aggregates, to give complex structures as micelles or large lamellar structures, whose size and shape depend on several factors including LPS concentration and osmotic pressure¹⁹ and therefore does not always reproduce the properties of the outer leaflet of Gram negative bacteria.



Other data, based on experiments carried out on bacterial cells with peptides active against Gram negative bacteria, support the idea that peptides form local aggregates on the outer leaflet, pass through the membrane by a self-promoted uptake, reach and cross the cytoplasmic membrane and finally interact with polyanionic targets such as DNA and RNA^{20,21}.

Recently published studies, carried out by time lapse fluorescence lifetime imaging on a melittin analogue with *E.coli* cells²², support the idea that melittin, unlike what has been demonstrated so far, does not form stable pores into the bacterial membrane, but instead it is uptaken by the cells through transient pores, reaches the inner leaflet causing leakage of the cell cytoplasm. Interestingly, in these studies there is no evidence of bacterial membrane breaking. Studies carried out in parallel on dipalmitoyl phosphatidylcholine vesicles show instead the formation of stable pores by the same peptide.

Such experimental evidences suggest that a more precise description of the mechanism of action of antimicrobial peptides requires studies performed in more complex systems as whole bacterial cells. We explored the use of CD for the determination of the secondary structure of antimicrobial peptides upon interaction with *E.coli* cells. We focused our studies on cecropin A and magainin 2, which are known to be random coils in buffer and fold into helices upon interaction with LPS, detergents or vesicles^{7,23–26}. Studies carried out in the presence of *E.coli* cells and LPS will allow to understand whether LPS represents a good model system to determine the secondary structure of peptides in the presence of bacterial cells.

Results

As far as we know, CD studies to detect the interaction of AMPs with bacterial cells have never been reported. In order to obtain reliable spectra we had to set the initial conditions for our experiments, investigating different buffers, peptide and cell concentrations.

CD set up: buffer, cell and peptide concentration. We initially recorded CD spectra in PBS buffer, since it is not toxic to cells and osmolar with cell cultures; this buffer has been employed in the past to study the interaction of cytotoxic drugs with eukaryotic cells²⁷. Unfortunately, this choice did not result in successful experiments, as the high tension voltage (HT) value was too high in the wavelength range we investigated, to allow further measurements. 10 mM phosphate buffer was chosen instead. In this buffer we verified the ability of *E.coli* bacterial cells to survive, either measuring the optical density at 600 nm (OD_{600}) within 6 hours and evaluating the ability of cells incubated in the buffer for 2, 4 and 6 hours to replicate once resuspended in LB (Figure S1). Interestingly, in phosphate buffer, cells are able to grow, as demonstrated by the increase in the OD_{600} recorded after 2, 4 and 6 hours of incubation; when cells incubated in phosphate buffer for 2, 4, 6 hours were centrifuged, resuspended in LB and shaken at 37°C for 90 minutes we also observed an increase in the OD_{600} with respect to that measured after dilution in LB, confirming that in phosphate buffer most of the cells survive and are able to recover. Finally we looked for the optimal cell concentration (measured as the OD_{600}) at which we could record good quality spectra and we could also detect elements of secondary structure in the peptides. To this aim we performed titrations of cells, resuspended in buffer at different concentrations, into the peptide solutions. We added cells aliquotes of 1–200 μ L from cell suspensions of 0.8 OD and 2 OD and we found changes in the CD spectra of the peptides after addition of aliquotes from the 2 OD cell suspension (Figures 1 and S2). In another set of experiments we added the peptides to solutions of cells at 0.1 OD and followed the changes of the CD signals with the time (Figure 2). CD spectra of *E.coli* cells show one broad negative band around 227 nm and also a shoulder around 210 nm, due to all the components of the cells. The intensity of the CD band enhances with the time when we compare samples at the same cell concentration, and also with increasing cell

amounts, when we compare samples incubated for the same time (Figures S3 and S4). We obtained good spectra at peptide concentrations of 5 μ M. At higher peptide concentration a bigger amount of cells seems to be required to induce the folding, but this is not compatible with the CD experiments.

Interactions of peptides with *E.coli* cells. Two sets of experiments were carried out. In the first set we added to the 5 μ M peptide solution (2.8 mL) 84, 112, 140, 168 and 196 μ L aliquotes of cells at 2 OD; we observed an increase in the intensity of the band around 222 nm at a fixed incubation time which is cell concentration dependent (Figure S5) for the cells + peptides mixtures. For cecropin A we found at the highest cell concentration tested a decrease in the intensity of the signal, likely due to a partial precipitation of the peptide.

Changes in the secondary structure of peptides were monitored, recording spectra after 0, 2, 4, 6 and 20 hours incubation of the peptides with cells: CD spectra of peptides + cells mixtures, immediately after the addition of cells, show very shallow minima around 207 and 222 nm, which become deeper after two hours of incubation (Figure S6). Spectra recorded at 4 and 6 hours display shallower minima in the same positions (Figure S6). Subtraction of the CD spectra recorded for cells to CD spectra of the cells + peptides mixtures, to eliminate the contribute of the CD signature of the cells, resulted in less intense CD spectra (subtracted spectra), showing one deep minimum around 207 nm and a shoulder around 224 nm, while the CD spectra of the peptides in buffers have one deep minimum at 200 nm (Figures S2 and 2). These data suggest that peptides change secondary structure after interaction with bacterial cells. The subtracted spectra account for the secondary structure of the peptides assuming there are no changes in the CD signature of the cells, other than those caused by incubation of cells in buffer. CD spectra are reported using as measurement units mdeg vs nm instead of molar ellipticity vs nm, as we cannot transform the CD signal for cells from mdeg to molar ellipticity.

Subtraction of the cell CD spectra to the cell + peptide CD spectra was carried out always zeroing all the spectra at 260 nm.

The intensity and position of the CD bands change with the amount of cells added: increasing the amount of cells at a fixed incubation time, the intensity of the band around 222 nm increased (Figure 1). A significant change in the peptide secondary structure is observed after 2 hours incubation with cells (Figure S2), although the content of secondary structure seems to be low.

Better results were obtained in a second set of experiments where we prepared solutions of cell in buffer at 0.1 OD (this concentration is identical to that obtained adding 140 μ L of cells withdrawn from the 2 OD suspension in 2.8 mL of peptide solutions) and added the peptides to a final concentration of 5 μ M. On such preparation we carried out time course experiments, recording spectra every 20 minutes for the first 2 hours and two more spectra after 4 and 6 hours of incubation. In the first 2 hours, if we look at the spectra of the peptides + cells mixtures, we observe minima around 209 and 224 nm, whose intensity increase with the time for both peptides (Figure S7a and b). If we compare the spectra of the mixtures at 2, 4 and 6 hours of incubation we see a similar pattern of bands, but a light difference in the intensity of the minima at 2 and 4 hours, with cecropin A showing the deepest bands after 2 hours and magainin 2 showing the deepest bands after 4 hours (Figure S7c and d). We also obtained spectra subtracting the CD of the cells (shown in Figure S8) to the CD of the mixtures cells + peptides (shown in Figure S7). The subtracted spectra are shown in Figure 2. In all cases the peptides evolve from random coils to helices following slightly different pathways; the bands are centered around 208 nm and 225 nm. In order to follow how the intensity of the CD bands changes with the time, we plotted the intensity of the CD signal at 222 nm vs time (Figure 3).

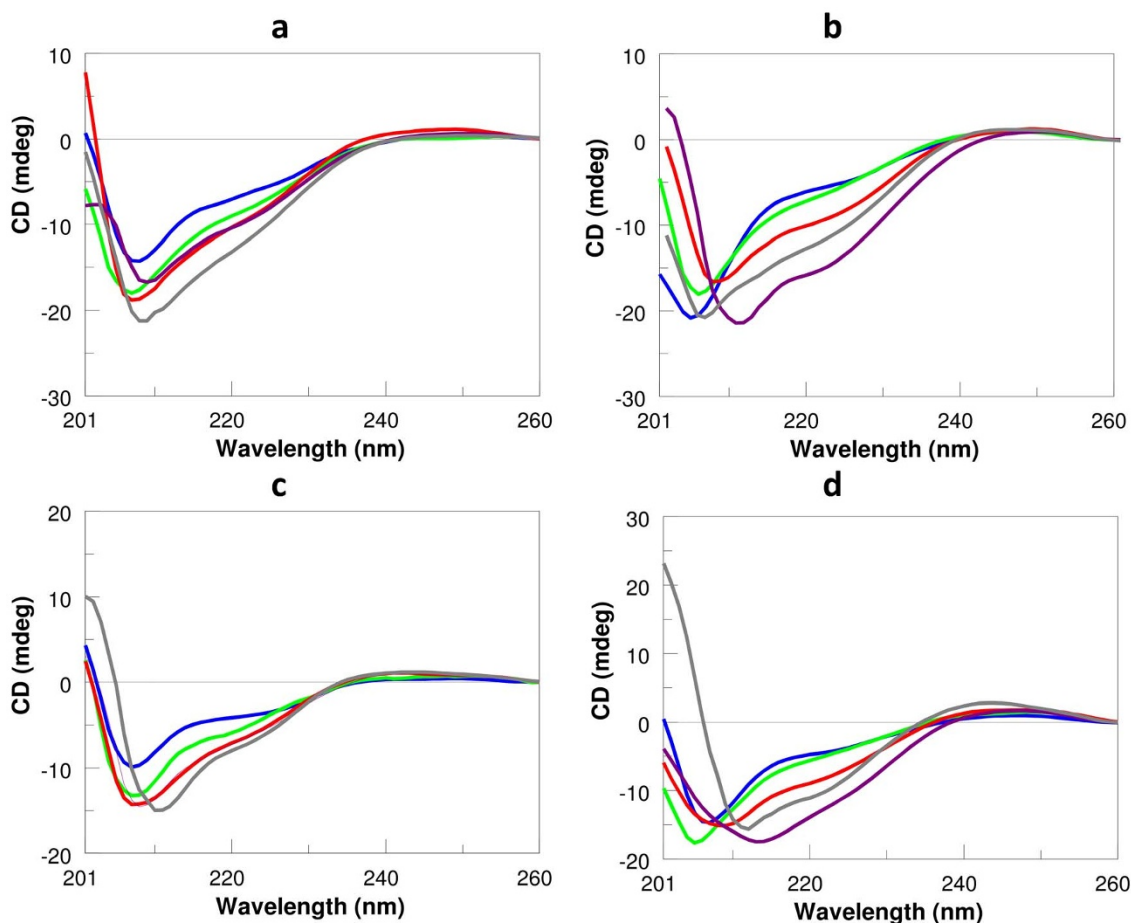


Figure 1 | Superimposition of CD spectra recorded at a fixed incubation time of the peptides (5 μM) with different amounts of *E. coli* cells in phosphate buffer (blue: 84 μL , green 112 μL , red: 140 μL ; purple 168 μL ; grey 196 μL). (a) magainin 2, 2 h incubation; (b) cecropin A, 2 h incubation; (c) magainin 2, 4 hours incubation, (d) cecropin A, 4 h incubation. Aliquotes of cells were withdrawn from suspension of cells at 2 OD. These spectra are representative of one set of experiments and are obtained after subtraction of the CD contribute of *E. coli* cells incubated for the same time as peptides in buffer.

The minima at 222 nm are reached, in these conditions, after 80–100 minutes incubation.

Finally, to demonstrate that the peptides fold upon interaction with the *E. coli* outer membrane components, we incubated the peptides with solutions obtained after sonication and centrifugation of 0.1 OD cell suspension, as those should contain the intracellular components of *E. coli*. CD spectra recorded for a 6 hours time period revealed that the structure of the peptide is always a random coil (Figure S9).

Interactions of *E. coli* LPS with peptides. We titrated aliquotes of LPS into peptide solutions, exploring LPS/peptide molar ratios from 0.1/1 to 2/1. CD spectra were also recorded titrating *E. coli* LPS into the buffer, to monitor the contribution of LPS to the spectra observed for peptides. Spectra of the LPS were subtracted to the spectra of the LPS + peptide mixture to eliminate the CD contribute of LPS. Subtracted spectra were less intense as compared to the LPS + peptide mixtures spectra. In all cases we found that addition of LPS to cecropin A and magainin 2 solutions resulted in a change in the secondary structure of the peptides; appearance of CD bands around 207 and 222 nm suggests that the peptides fold into alpha helices. Increasing amounts of LPS result in CD spectra with deeper minima (Figure 4 a and b).

Time course experiments were performed also on peptide/LPS mixtures at a 1:1 molar ratio; the plot of the intensity of the CD signal at 222 nm vs the time shows that the CD signal decreases with the time, reaching a plateau after about 2 hours (Figure 4c and d).

Discussion

We explored the interaction of Gram negative bacteria cells with antimicrobial peptides using CD to gain information on the secondary structure the peptides assume once they meet the bacterial cells; the results obtained, together with data reported in the literature, allow us to add some details on the mechanism of action of the antimicrobial peptides. These studies were carried out as lipid mixtures and LPS, which are currently employed in place of bacterial cells, do not carefully reproduce the environment of bacterial cells, which is in comparison a more dynamic and chemically complex system.

Studies were carried out on two well characterized antimicrobial peptides, namely cecropin A and magainin 2 in the presence of *E. coli* cells; we also investigated the secondary structure of the peptides incubated with *E. coli* LPS. These latter experiments were performed with the aim to compare results obtained in the two different experimental conditions and to assess whether purified LPS is a good model system to reproduce the Gram negative bacteria environment.

Magainin 2 and cecropin A are active against Gram negative and Gram positive bacteria and have been demonstrated to interact with the outer leaflet of bacterial cells^{23–25,28–30}. These peptides are highly membrane active and their mechanism of action has been hypothesized by different biophysical techniques. We wish to highlight that different techniques yield results which are interpreted in different ways.

Magainin 2 forms an amphipathic helix in organic solvents and dodecylphosphocholine (DPC) micelles; fluorescence and solid state

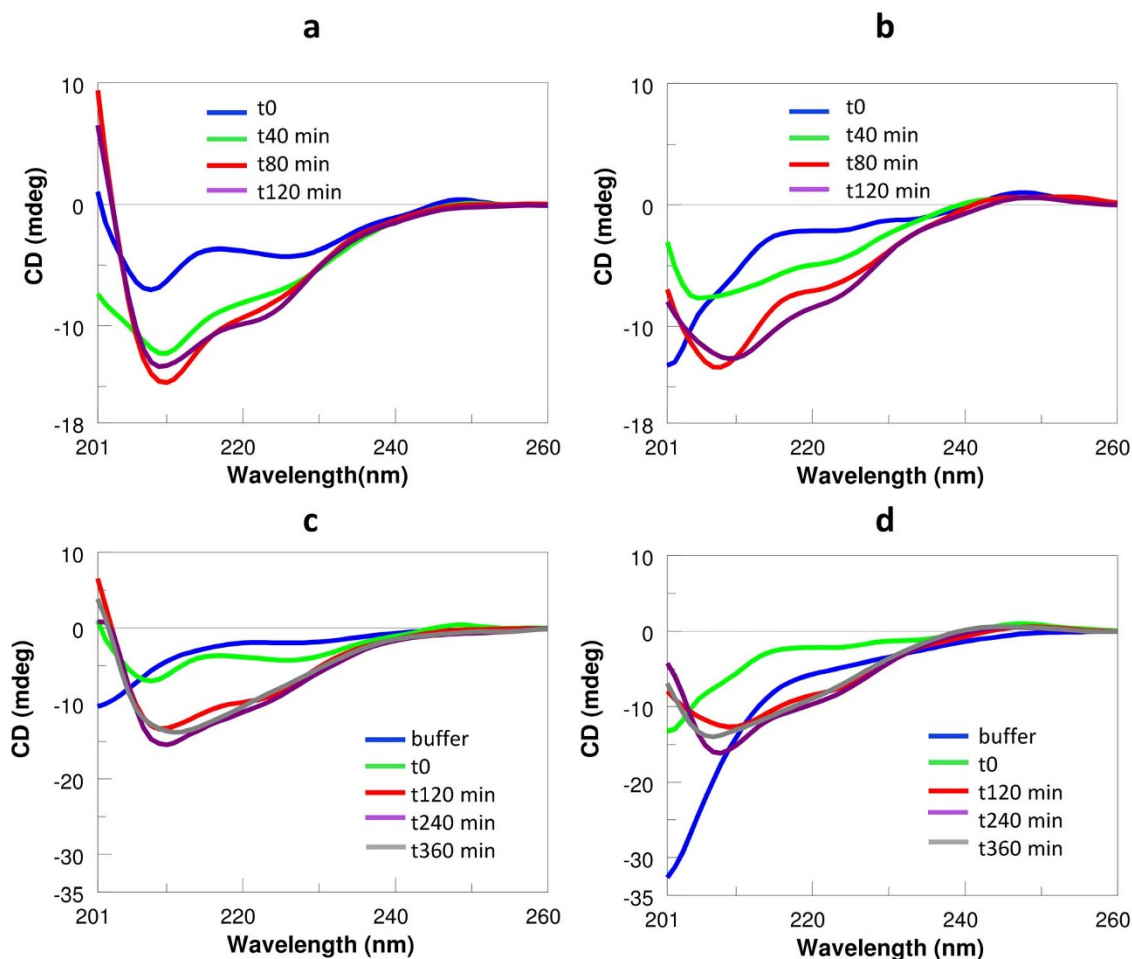


Figure 2 | Superimposition of the CD spectra obtained incubating *E.coli* cells at 0.1 OD with 5 μM peptides at different times. Panels a and b report spectra respectively for magainin 2 and cecropin A in a 2 hours time period; panels c and d show spectra of the peptides (magainin 2 panel c and cecropin A panel d) after incubation times of 2, 4 and 6 hours. These spectra are representative of one set of experiments and are obtained after subtraction of the CD contribute of *E.coli* cells incubated for the same time as peptides in buffer. The spectra of the peptides in buffer (without cells) are reported in panels c and d.

NMR studies have demonstrated that the peptide first lies parallel to the membrane surface, successively forms a toroidal pore and likely translocate across the bilayer^{9,25,31}. Experiments carried out on *E. coli* cells demonstrated that addition of magainin 2 to bacterial cells

caused spheroplast lysis, K^+ ion leakage and cell death. Interestingly, the leakage of β -galactosidase was reported to be undetectable, suggesting that the peptide does not induce large lesions into the bacterial membrane³².

Cecropin A has been demonstrated by IR experiments in dimyristoylphosphatidylcholine (DMPC) and by NMR run in hexafluoroisopropanol to fold into a helix^{10,23}. Solid state NMR studies carried out with DMPC/dimyristoyl phosphatidylglycerol (DMPG) bilayers suggest that cecropin is oriented parallel to the membrane surface; in this experimental setting, pore formation, which has been hypothesized by electrical conductivity measurements on lipid bilayers, is not detectable³³. Interactions of cecropin A with *E.coli* induce a transcriptional profile response of the bacteria³⁴.

In this paper, as far as we know, we report for the first time studies by CD on the secondary structure of antimicrobial peptides in the presence of *E.coli* cells; these studies rely on some approximations due to the experimental conditions in which measurements are performed. In particular, cells are suspended in phosphate buffer which is compatible with CD measurements and with cell survival (Figure S1); we assume that in this condition the overall CD signature of the bacterial cells is independent on the presence of the antimicrobial peptide, although we hypothesize that cells undergo a change due to leakage and lysis (Figure S10). Furthermore, although we measure the cell concentration every time before using the cell solutions, we have to consider that the growth of cells in buffer proceeds all the

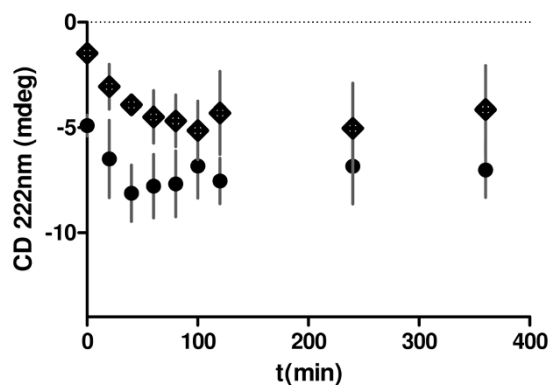


Figure 3 | Variation of the CD signal at 222 nm vs time obtained subtracting the cell signal to the cells + peptides mixtures signal. *E.coli* cells at 0.1 OD were incubated with the peptides cecropin A (squares) and magainin 2 (circles) for 6 hours. Points are obtained as the mean (\pm SEM) of three experiments.

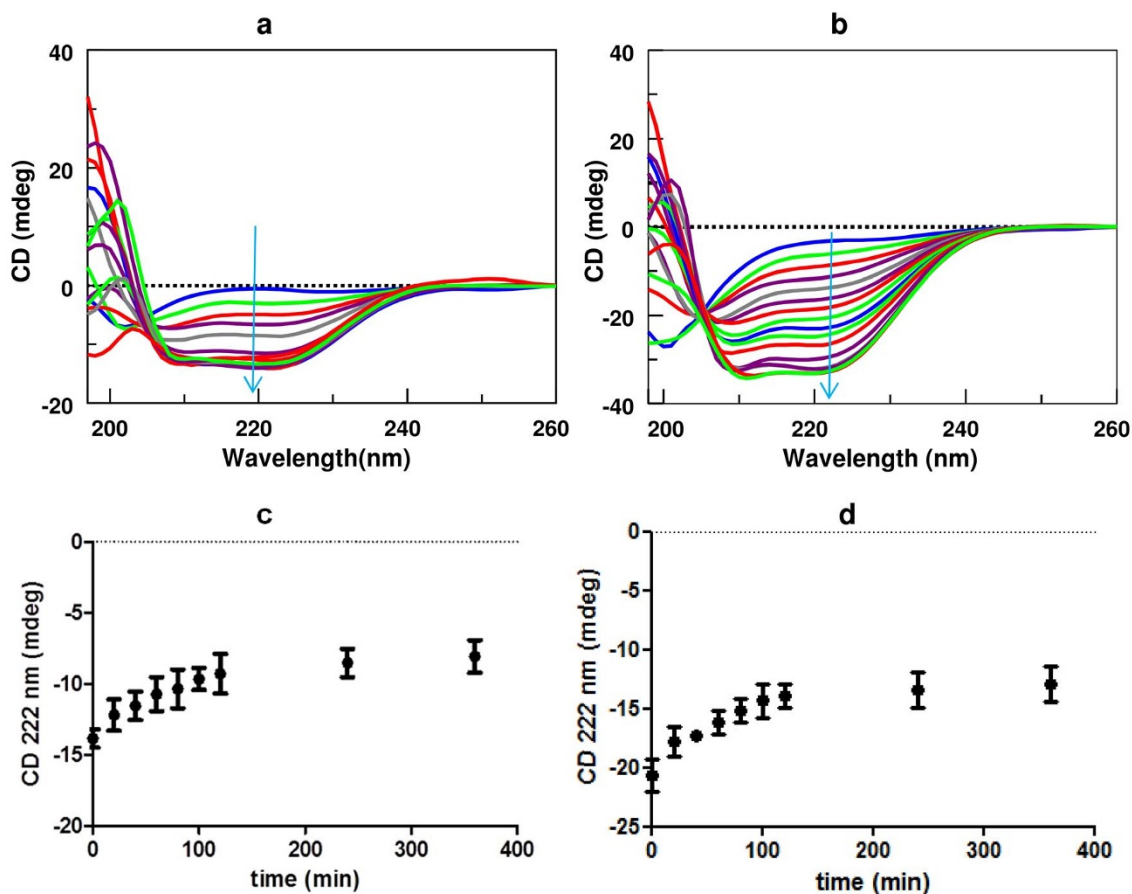


Figure 4 | Superimposition of CD spectra recorded for magainin 2 (5 μM) (a) and cecropin A (5 μM) (b) at increasing *E. coli* LPS concentration, as indicated by the arrow. These spectra are representative of one set of experiments. Variation of the CD signal at 222 nm recorded within 6 hours for peptides (5 μM)/LPS mixtures at 1 : 1 molar ratio for magainin 2 (c) and cecropin A (d). The CD spectra of LPS were subtracted.

time in a slightly different way. This means that different batches of cells will produce CD spectra which are not superimposable, but very similar in shape. We recorded CD spectra of cells and of peptides + cells mixtures at different cell concentrations and at different times (Supporting information). We obtained also spectra of the peptides after subtraction of the spectra of the cells to highlight the secondary structure of the peptides. We will discuss here subtracted spectra.

The shape of the spectra depends on the cell concentration, on the time the peptides were incubated and also on the protocol employed to prepare the mixtures of cells + peptides. Interestingly when the peptides are added to the cell solution, we obtain better spectra as compared to those obtained when the cells are added to the peptide solution (compare Figures S2 and 2). For cecropin A, the comparison of the spectra recorded after mixing the peptides with different aliquotes of cells reveals that the secondary structure is cell concentration dependent: increasing the cell concentration the depth of the minimum around 222 nm increases (Figure 1). A very similar trend is found for magainin 2 (Figure 1). These results suggest that the ability of the peptides to assume secondary structure is related to the cell concentration: at low cell concentration likely a higher content of random coil over helical peptide is present. This result may be due to the concentration of LPS available to bind the peptide, which increases with cell concentration. When we look at the time course experiments (Figure 2), the position of the CD bands clearly indicates that the peptides fold into helices upon interaction with cells. For both peptides, spectra obtained immediately after mixing the cell suspension with the peptide solution show signals weaker as compared to those observed even after 20 minutes of incubation

(Figure 2). These data suggest that the folding of antimicrobial peptides is not a very fast process, likely antimicrobial peptides first adhere to the outer leaflet of bacterial cells and then fold. After 80–100 minutes of incubation of the peptides with the cells, the CD spectra of the peptides reach their deepest minima (Figure 3). We also know that, in our experimental conditions, within this time, cells leak and reasonably are almost all lysed by the peptides (Figure S10). After this time the intensity of the CD bands is very variable, likely due to a change in the concentration of the peptide in the solution. This effect can conceivably be interpreted hypothesizing that cell lysis is followed by a partial precipitation (which is not visible) of peptides attached to cells. CD spectra recorded in the presence of the intracellular components of *E. coli* cells (Figure S9) demonstrate that the folding of the peptide is induced by the components of the bacterial outer membrane.

When LPS is titrated into peptide solutions we observe CD bands around 207 and 222 nm, whose intensity depends on LPS concentration (Figure 4). At very low LPS concentration the peptides fold into alpha helices; the position of the bands does not change much at increasing cell concentration, the intensity of the 222 nm band reaches a plateau at a high peptide/LPS molar ratio. These results, compared to what obtained with cells, demonstrate that the folding of the peptides is induced by LPS. The difference in the position of the bands might be due to a subtle difference in the secondary structure of the peptide in the presence of cells and LPS or to artifacts deriving from operations like subtraction of spectra. We have to recognize that the comparison of CD spectra obtained with *E. coli* LPS and cells is qualitative, as we do not know the concentration of LPS on



bacterial cells; also we are comparing two systems (LPS and cells) with different chemical composition, different structural organization and size. The time course experiments performed with the peptide/LPS mixtures suggest that even with LPS, the binding and folding processes are followed by precipitation of the peptides or of the complexes between the peptides and LPS.

In conclusion, the results described demonstrate that CD is a technique suitable for studying the interaction of peptides with *E.coli* cells. In the context of studies on the interactions of antimicrobial peptides with cells, we show that the folding of peptides as magainin 2 and cecropin A on *E.coli* cells:

- is not a fast process but occurs within two hours;
- is driven by the components of the outer membranes of *E.coli* bacterial cells.

Methods

Magainin2 and cecropin A were purchased from Anaspec Inc. and Bachem; LPS of *E. coli* 0111:B4 (purified by gel filtration chromatography) was purchased from Sigma Aldrich and used without further purification. BL21 (DE3) cells were purchased from Novagen. CD Spectra were recorded on a spectrophotometer Jasco J-715.

Cell culture. *Escherichia coli* BL21 (DE3) cells were grown in LB medium at 37°C, harvested while in exponential phase (OD₆₀₀ nm: 0.6–0.8), centrifuged (5000 rpm, 10 min, 4°C) and resuspended in 10 mM phosphate buffer pH 7.0 at OD₆₀₀ of 2.0 or 0.1.

Cell survival assay. Two different assays were carried out in order to test cells survival:

- Cell survival assay was carried out diluting 100 µL of cell suspension with an optical density of 2 in 1.0 mL of 10 mM phosphate buffer, pH 7.0 and monitoring cells growth every two hours (2 h, 4 h and 6 h) at room temperature, measuring OD₆₀₀.
- 280 µL of cell suspension with an optical density of 2 were incubated in 5.6 mL of 10 mM phosphate buffer pH 7.0 for 2 h, 4 h and 6 h. Then cells were centrifuged (5000 rpm, 10 min, 4°C), diluted in LB medium (5.6 mL) and incubated at 37°C for 90 min. The OD₆₀₀ after dilution in LB and after incubation was observed.

All experiments were repeated in duplicate.

Circular Dichroism. CD spectra were recorded at 25°C using a 1 cm quartz cell using a 260–201 nm measurement range, 100 nm/min scanning speed, 1 nm bandwidth, 4 s response time, 1.0 nm data pitch. Peptides concentration for CD measurement was 5 µM in 10 mM sodium phosphate buffer, pH 7.0. Peptide concentration was determined measuring the absorbance at 280 nm for cecropin A ($\epsilon_{280} = 5500 \text{ M}^{-1} \text{ cm}^{-1}$) and at 205 nm for magainin 2 ($\epsilon_{205} = 94390 \text{ M}^{-1} \text{ cm}^{-1}$)³¹.

Peptides and LPS. LPS titrations were carried out recording the CD spectra of the peptides (magainin 2 and cecropin A) in the presence of increasing concentrations of LPS at 25°C, up to a molar ratio LPS/peptide of 2:1. The peptides were dissolved in phosphate buffer 10 mM pH 7.0 at a 5 µM concentration; the LPS was dissolved in phosphate buffer 10 mM pH 7.0 at a 50 µM concentration, before use it was subjected to temperature cycles between 4° and 70°C, interrupted by vortexing (10 min), as described elsewhere¹². The sample was stored at 4°C overnight. Spectra of the mixtures LPS + peptides and LPS were recorded. Spectra are shown after subtraction of the LPS CD spectra to the LPS + peptide mixtures CD spectra.

The time course experiments were performed using mixtures of 5 µM peptides and 5 µM LPS. CD spectra were recorded every 20 minutes for 2 hours, two more spectra were recorded after 4 and 6 hours. A plot of the intensity of the CD signal at 222 nm vs incubation time was obtained, the signal of LPS was subtracted.

All experiments were repeated in duplicate.

Peptides and cells. The variation in peptide secondary structure during the time was monitored.

First set of experiments. The peptides were dissolved in phosphate buffer 10 mM pH 7.0 at a 5 µM concentration; cells were resuspended in phosphate buffer 10 mM pH 7.0 at 2.0 OD. Different amounts of cells (84 µL, 112 µL, 140 µL, 168 µL, 196 µL) were added to peptide solutions (2.8 mL) and CD spectra were recorded every two hours (2 h, 4 h, 6 h and 20 h) at 25°C.

Second set of experiments. A solution of cells in phosphate buffer pH 7.0 at 0.1 OD was prepared. To this solution peptides were added to a final concentration of 5 µM. CD spectra were recorded every 20 minutes for 2 hours, two more spectra were recorded after 4 and 6 hours incubation.

All the experiments were carried out at least in duplicate.

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Acknowledgements

The author are grateful to Dr. Lucia de Rosa for helpful discussion and help with cell culture. C.A. is granted by Programma Merit RBNE08YFN3.

Author contributions

C.A. performed the experiments, L.D.D. and A.R. designed the experiments and wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Avitabile, C., D'Andrea, L.D. & Romanelli, A. Circular Dichroism studies on the interactions of antimicrobial peptides with bacterial cells. *Sci. Rep.* **4**, 4293; DOI:10.1038/srep04293 (2014).



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