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The role of macromolecular crowding in single-entity electrochemistry: friend or foe?

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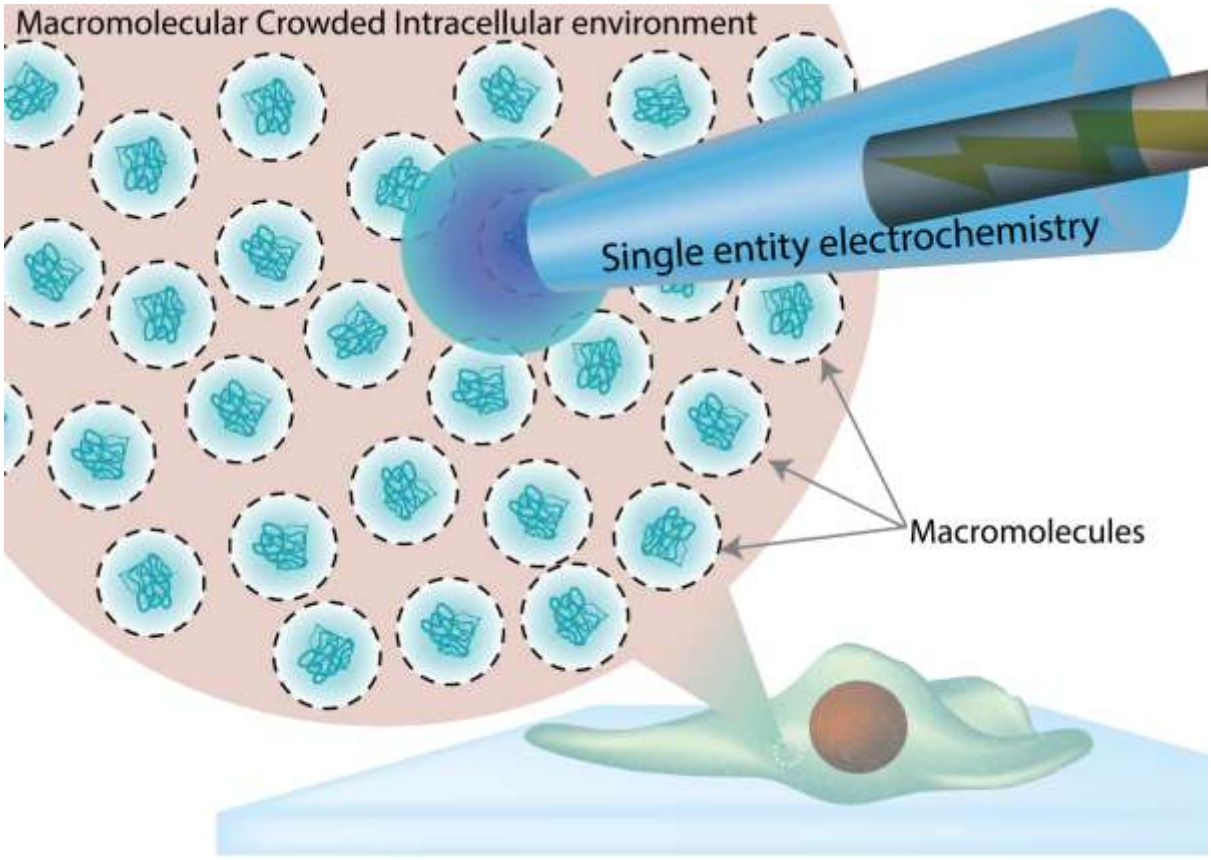
1. Highlights

- The intracellular environment is macromolecularly crowded. Such environment plays an important role in regulating cellular physiology.
- Single entity electrochemistry enables high resolution intracellular measurements. However, the effects of the intracellular crowded environment on the measurement is often neglected.
- Recent reports showed that *in vitro* macromolecular crowded environment causes a signal enhancement on the detection of single entities with nanopores and nanoelectrodes.

2. Abstract

The recent development of nanoscale probes has enabled the study of single molecules and single cells with unprecedented resolution and the expansion of the field of single-entity electrochemistry. There is a growing of evidence suggesting that highly crowded intracellular environment facilitate nanoelectrochemical measurements in cells by improving the signal-to-noise ratio. In this opinion piece, we discuss the concept of macromolecular crowding and its implications in single-entity electrochemistry.

3. Graphical Abstract



4. Keywords

macromolecular crowding; cell biology; electrochemistry; single-entity; nanopipette; single-molecule; nanopore

5. Introduction

Nanoelectrochemical methods [1, 2], have great potential to perform the analytical detection of biomolecules and metabolites both in and around living cells and tissues [3-14] and the recent development of nanoscale probes has enabled the study of single molecules and single cells with unprecedented resolution and the expansion of the field of single-entity electrochemistry [15-17]. For example the dual-barrel carbon nanopipette allowed the high resolution topographical and electrochemical mapping of the membrane of living cells [18] and further development of this technology permitted the intracellular measurement of electrochemically active metabolites [19, 20]. Pan *et al.* (2020) demonstrated that nanopipettes can be employed to carry out resistive pulse detection of vesicles within living cells [21]. However, these studies represent only few examples of the numerous applications of nanoelectrochemical techniques for the single cell analysis and the development and application of these technologies for intracellular measurements have recently been summarised [15, 16].

In this opinion piece we will discuss macromolecular crowding and highlight recent work that demonstrates its impact on the sensitivity of single molecule sensing. We will also discuss the possibility that electrochemical detection can be enhanced by macromolecular crowding and how the crowded intracellular environment may affect the intracellular detection of biomolecules.

6. Macromolecular crowding and its impact on the behaviour of biomolecules

In general terms, a macromolecular crowded environment is defined when 20% or more volume of a solution is occupied by macromolecules, leading to a reduced accessible volume to the molecule of interest (Figure 1) [22]. The intracellular environment is highly crowded and contains a high concentration of macromolecules. Both prokaryotic [23] and eukaryotic cells

[24] contain on average 300 g/l of macromolecules tightly packed within their intracellular environments. This equates to over 30% of the cellular volume being occupied by macromolecules.

The effect of molecular crowding is often neglected when biological macromolecules are studied *in vitro.* However, macromolecular crowding is known to affect numerous intracellular activities. One prominent example being the difference in the enzyme reaction kinetics and the substrate association rate observed inside the cell when compared to the 'test tube' experiment [25]. Alteration it the kinetics and association of a single enzyme may not cause any difference, small changes in several enzymes can affect the overall metabolic activity of a cell, as the metabolic activity of a cell is contributed by networks of interconnected enzymes and substrates, this can cause significant deviation from the 'test tube' measurement [26, 27]. It has also been suggested that the cell volume regulation response is a by-product of cytosolic macromolecular crowding, where the cell reduces the liquid volume to modulate cellular physiology such as promoting structural reorganization and reduce protein transport [28]. Scientists have therefore attempted to replicate the macromolecular crowded environment of the cell *in vitro*. Inert neutral synthetic polymers such as polyethylene glycol (PEG), dextran and Ficoll are commonly used *in vitro* as macromolecular crowders [25, 29]. Alternatively, proteins such as bovine serum albumin (BSA) and lysozyme, or cell lysates, can be used to form a macromolecular crowded environment [25, 29, 30].

The effects of macromolecular crowding are fairly simple to appreciate when only steric repulsion is considered. In simple terms, a molecule of interest in a macromolecular crowded environment must avoid steric overlap with other macromolecules, since this would be energetically unfavourable [31]. As a result, the accessible volume for the molecule of interest directly depends on the amount of volume occupied by the macromolecular crowders, this is termed as the excluded volume effect and it is the first and most important effect caused by the crowders on the molecule of interest [32, 33] (Figure 1A). The second effect on the molecule of interest is its diffusion hindrance. An increase in the crowder concentration affects

the diffusion of the molecule of interest with its magnitude depending on the properties and the size of the crowders. When the crowders are smaller and more mobile than the molecule of interest, the molecule of interest can diffuse freely but more slowly due to the increased viscosity of the solution. However, when the crowders are comparable in size or larger than the molecule of interest and relatively immobile, the molecule of interest is constrained into a limited space, an effect known as the anomalous diffusion (Figure 1B) [32, 34]. The molecule of interest undergoes anomalous diffusion and no longer follows Brownian diffusion. In this instance the mean square displacement change over time is typically characterized by a sublinear increase, and this describes the diffusion behaviour of macromolecules and organelles inside cells [35, 36]. Lastly, the molecule of interest always adopts the most compact state to reduce the occupied volume in the highly crowded environment, this is due to the excluded volume effect, as depicted in other studies investigating the dynamics of polymers in a confined space [37-39]. Placing another molecule of interest in the same space inevitably increases the binding affinity between these molecules, this interaction is termed depletion interaction and is affected by the strength of the excluded volume effect, and the diffusion hindrance (Figure 1C) [32, 40].

These effects could have a strong impact on the electrochemical measurement, for example the diffusion hindrance could affect the interpretation of electrochemical data that often rely on diffusion coefficient calculated in the uncrowded condition. The sensor efficiency maybe altered due to depletion interaction which consequently promotes a stronger interaction between the sensor and the molecule of interest. Extensive reviews on the effects of macromolecular crowding on the behaviour of biomolecules can be found here: [25-27, 29, 30, 32, 41-43].

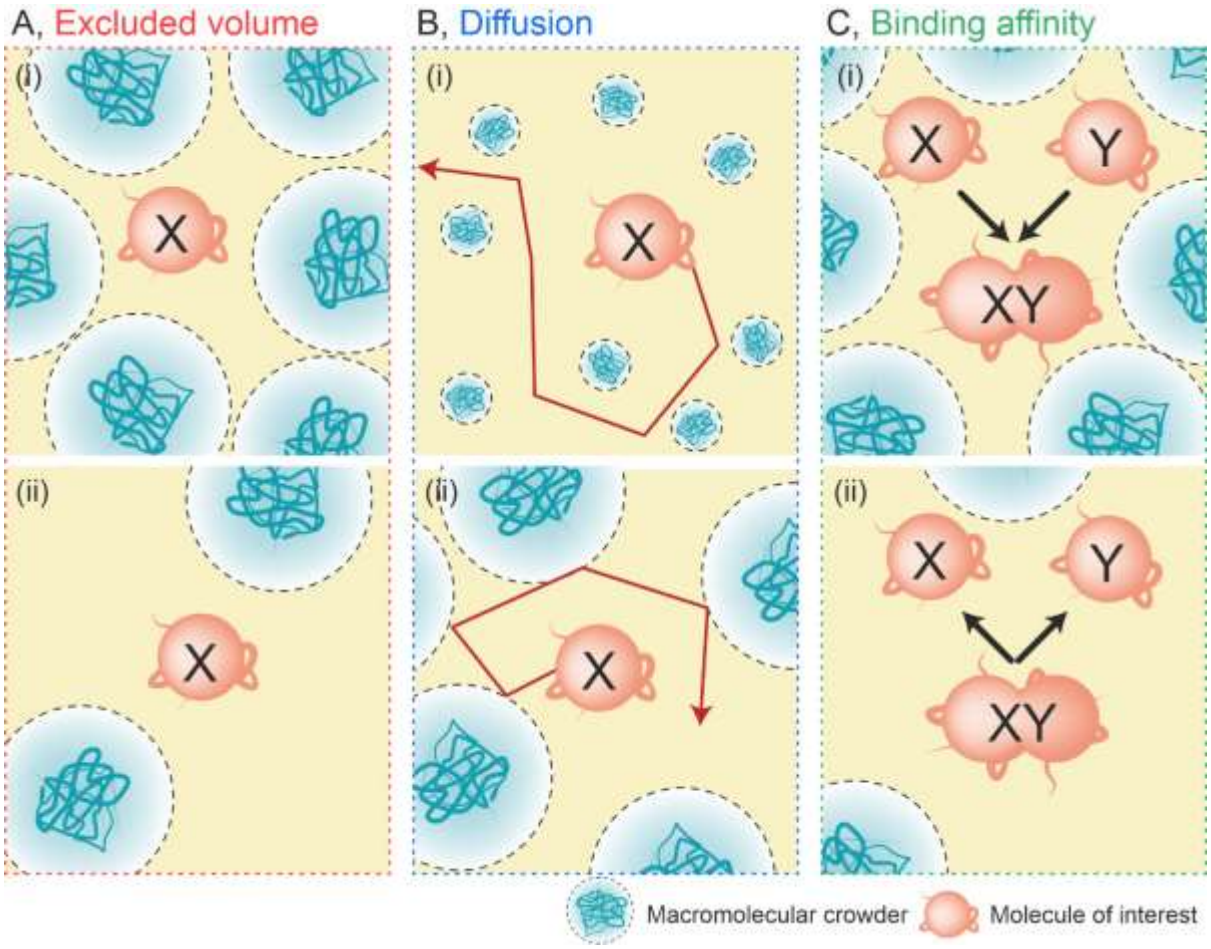


Figure 1. The effects of macromolecular crowded environment on the molecule of interest. (A) The molecule of interest X is placed either in the highly crowded (i) or uncrowded (ii) environment. The access volume (yellow background) is reduced in the highly crowded environment compared to the uncrowded environment. (B) The diffusion of the molecule of interest X is moderated by the size and the properties of the crowders. Smaller mobile crowders do not hinder or constrain the molecule of interest X and it freely diffuses throughout the volume (i). Larger immobile macromolecular crowders hinder and constrain the diffusion of the molecule of interest X, causes it to undergo anomalous diffusion (ii). (C) The binding affinity of the molecules of interests under the highly macromolecular crowded (i) or the uncrowded (ii) environment. The highly macromolecular crowded environment causes the molecule of interest X to adopt a more compact state, placing another molecule of interest Y in the same space inevitably causes them to interact to form a new complex XY via the depletion interaction. However, when the environment is uncrowded, the molecules of interest are less likely to interact and form a complex.

7. Utilisation of Macromolecular crowding in single molecule detection

Macromolecular crowding is an important factor that is often neglected in single entity electrochemistry [1]. Recent evidence suggests that the translation of nanoelectrochemical methods from *in vitro* to the crowded intracellular environment affects the performance of the technology [21]. Similarly, multiple groups working with nanopore-based platforms have studied the effect of PEG induced macromolecular crowding on the resistive pulse measurement of single molecules through a nanopore [44-46].

Earlier studies utilized PEG to measure the nanopore size of biological membrane channels and to probe the behaviour of molecules inside the nanopore [47-52]. The macromolecular

crowder PEG is available in a wide range of different molecular weights and thus has different radii of gyration. It has been observed that PEGs with smaller gyration radii can freely enter the nanopore, but the larger PEGs are physically excluded [51]. However, when the concentration of PEG approaches 20% (w/v), the repulsion between the PEGs forces the larger PEG molecules to enter the nanopore. This observation can be explained by the depletion interaction phenomenon depicted in the last session, which promotes the PEG and the nanopore to interact more frequently than in uncrowded conditions [53-57].

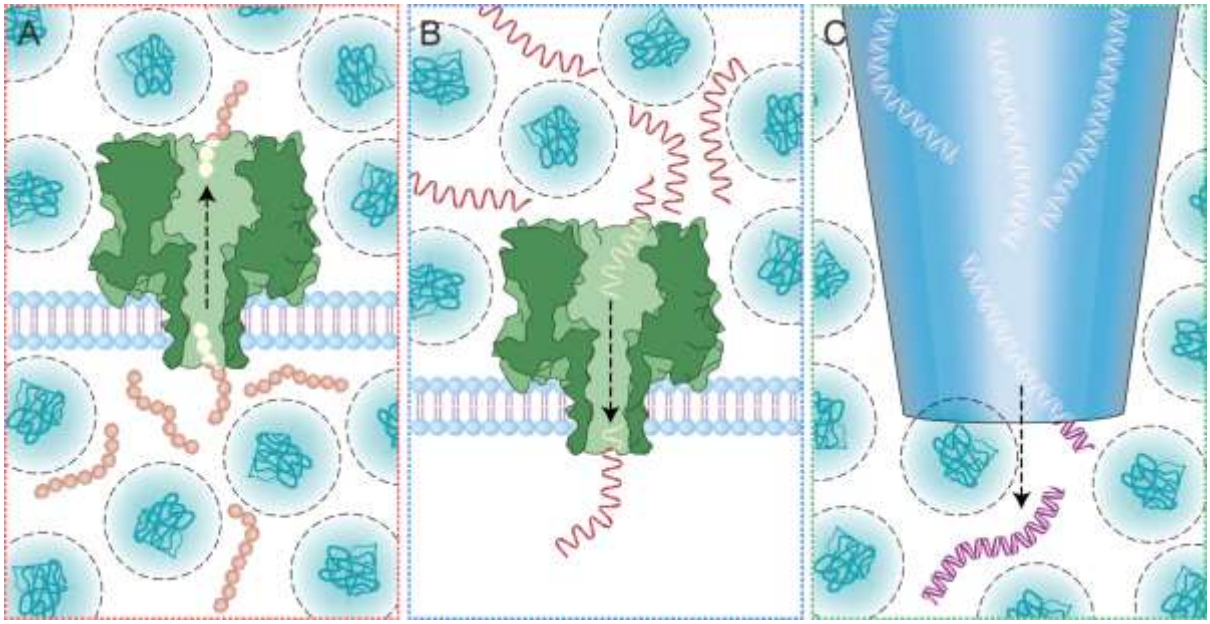


Figure 2. Three studies have used macromolecular crowding to enhance the detection of molecules. (A) PEG was at equal concentration on both sides of the nanopore to form a symmetrical macromolecular crowded environment. The detection of the 23 aa peptide Syn-B2 by α-HL was found to be enhanced when PEG 4000, and PEG 8000 were used at 25% (w/v). (B) 40% (w/v) PEG was mixed with a 92nt ssDNA and the ssDNA was translocated through the α-HL nanopore into PEG-free solution. (C) dsDNAs and proteins were translocated through a glass solid-state nanopore from a PEG-free solution into a macromolecular crowded PEG solution. (A) Redrawn from [44], (B) Redrawn from [45], (C) Redrawn from [46].

Inspired by the crowded intracellular environment and the observed alteration in the behaviour of the PEG at high concentrations, three studies, each in different ways, have utilised the macromolecular crowder PEG to enhance the single molecule sensitivity of nanopores [44-46]. Larimi *et al.* (2019) utilised a symmetrical macromolecular crowded environment, *i.e.* both sides of the nanopore contained an equal concentration of PEG. Using this they showed that the detection of the 23 aa long polypeptide Syn-B2 by an α-haemolysin (α-HL) nanopore was greatly enhanced [44] (Figure 2A). Later, Yao *et al.* (2020) utilised an asymmetric macromolecular crowder gradient where the 92 nt ssDNA was mixed with PEG, and translocated through the α-HL into the PEG-free solution (Figure 2B) [45]. These two groups both tested PEGs with different molecular weight at various

concentrations, and identified that the most pronounced increase in the sensitivity of the α-HL nanopore was with PEG 4000 at ≥ 20% (w/v) [44, 45]. Although PEG 4000 should be able to enter the nanopore at this high concentration, the electrophoretic force acting on the charged polypeptide and ssDNA would lead to higher probability of these molecules entering the nanopore than the neutral PEG. The mechanism behind the enhanced sensitivity for these biological macromolecules was attributed to entropic effects and the alternations in the kinetics and equilibrium of the interactions between the analyte molecules and the α-HL due to the highly crowded environment [32, 44, 45].

We recently demonstrated that macromolecular crowding can also improve the sensitivity of a solid-state nanopore for the detection of dsDNA and proteins [46]. We used a glass nanopipette with a 10nm nanopore at its tip as the model solid-state nanopore to deliver the biological macromolecules into a macromolecularly crowded environment generated by 50% (w/v) PEG 8000 (Figure 2C). Importantly, the enhanced sensitivity of the nanopipette was not solely due to the macromolecular crowding effects described above [44, 45]. In studies involving biological nanopores like α-HL, PEG 4000 was restricted from the nanopore due to the gyration radius being larger than the nanopore of α-HL. However, the PEG 8000 used in our study could enter the nanopore of the nanopipette as the nanopore diameter used was at least 4 times larger than the α-HL biological nanopore. Additionally, the depletion interaction between the nanopore and the analyte molecules mechanism may not occur here as the analyte molecules were driven from an uncrowded to a highly crowded solution. Finally, we observed an inversion of the current-voltage rectification of the nanopipettes at 50% (w/v) PEG 8000, but not at concentrations below 50% (w/v) for PEG 8000, indicating that the highly crowded environment also modifies the nanofluidic properties of the nanopipette [58].

Based on these observations, we believe it would be extremely interesting to study and elucidate the effects of macromolecular crowding on the detection sensitivity of intracellular molecules with nanoelectrochemical methods. Recently, Pan *et al.* (2020) employed a nanopipette to detect Au nanoparticles, ROS and RNS via the resistive pulse sensing technology within living cells [21]. Interestingly the authors observed a marked increase in the signal-to-noise ratio when performing measurements inside cells. This effect is remarkably similar to the effect we observed in vitro with macromolecular crowding by PEG 8000 on the detection of biological analytes [46]. The implication is that the macromolecular crowded environment of the cell may also enhance the sensitivity of detection of resistive pulse sensing by a nanopipette.

Macromolecular crowding has also been shown to improve the sensitivity of other electrochemical devices. Ricci *et al*. (2007) demonstrated for an electrochemical DNA sensor that increased surface crowding with DNA is linked to an improved signal-to-noise ration upon analyte binding, but this is at the expense of an increased time for the sensor to equilibrate [59]. Recently, Xie *et al.* (2020) developed a macromolecular crowded electrolyte using PEG as the macromolecular crowder to decrease water activity and improve the performance of a high voltage aqueous battery by improving the operating window with low salt concentration [60]. This study highlights that macromolecular crowding could have potential applications for the wider electrochemical community [61-63].

8. Conclusion

To date, a limited number of nanoelectrochemical studies have been performed in a macromolecular crowded environment and they have mostly been limited to the detection of single molecules with nanopores. However, these studies indicate that the alteration of the solution environment from uncrowded to crowded significantly enhances the sensor's sensitivity. With the increased application of *in vitro* [64] and *in vivo* [65] electrochemistry techniques, macromolecular crowding needs to be considered in order to accurately interpret the measured signals. Moreover, recent evidence suggests that highly crowded intracellular environment could facilitate electrochemical measurements in cells by improving the signal-to-noise ratio. In, addition to the study of biological systems, macromolecular crowding may also be used to improve sensor sensitivities and to benefit the wider electrochemical community and maybe become the electrochemist best friend.

9. Competing Interests

The authors declare no competing interests.

10. Funding

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12. Author Contribution

All authors wrote and corrected the manuscript.

13. Acknowledgements

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14. Abbreviations

Polyethylene glycol, PEG; α-hemolysin, α-HL; bovine serum albumin, BSA; reactive oxygen species, ROS; reactive nitrogen species, RNS; amino acid, aa; dsDNA, double stranded DNA; ssDNA, single stranded DNA.

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