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Effect of Metals on Underwater Adhesion of Gastropod Adhesive Mucus

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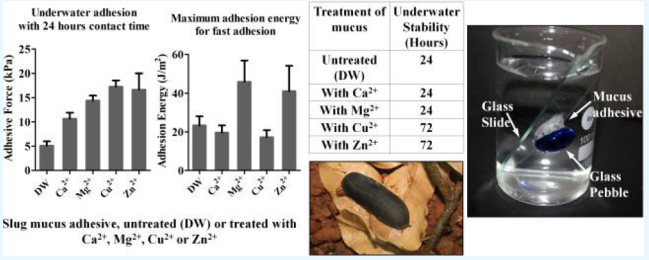
ABSTRACT: Many gastropods release mucus hydrogels, which help them to remain attached to different substrates. Although not as strong as synthetic or biomimetic adhesives, some of these hydrogels have the ability to adhere to wet surfaces. These complex hydrogels mainly consist of proteins and carbohydrates, their natural cross-linking reactions being dependent on the presence of metals. In this paper, we investigated the role of metals in improving the underwater adhesive property of the mucus hydrogel from the slug *Laevicaulis alte*. We found that the strength and duration of attachment of two glass surfaces under water by the mucus hydrogel could be enhanced by its simple treatment with salts of metals, namely, Ca, Mg, Cu, or Zn. The degree of enhancement followed the order Ca^{2+} < Mg^{2+} < Zn^{2+} < Cu^{2+}. The Cu^{2+}-treated hydrogel kept two glass surfaces attached under water for about 20 days, while Zn^{2+} treatment caused attachment for about 15 days, as compared to the 3−5 days of attachment caused by the untreated gel. Treatment with both metals increased the underwater stability of the hydrogel almost threefold, presumably by strengthening its cross-linking. However, the Cu^{2+}-treated hydrogel fell short of its adhesive function in the case of fast attachment within time scale of minutes, showing considerably low adhesive strength. From this study, we conclude that the treatment with Zn^{2+} is the best choice for improvement of the underwater adhesive property in terms of strength and stability. Overall, this work presents a novel biological underwater adhesive. The dynamic behavior of this multicomponent hydrogel in a versatile metal-rich environment may guide us toward designing new useful biomimetics.



INTRODUCTION

Several marine organisms secrete adhesives that were found to be effective under water. Some of the well-known examples are mussel adhesive plaques, barnacle cement, sandcastle glue, and caddish fly larvae. Their adhesive properties have been attributed to specific proteins, such as adhesive proteins containing phosphorylated serine in sandcastle glue, mussel adhesive proteins containing the modified amino acid DOPA, barnacle highly charged cement proteins, etc.[1,2] These adhesive proteins, directly acquired from nature and effective in a wet environment, were the obvious choice of materials for designing tissue adhesives. However, obtaining these proteins in adequate quantity has been a challenge. Therefore, various biomimetic adhesives have been designed based on the mechanisms of their adhesive interactions. Many of these works reported the use of phosphate, DOPA, or amine group-functionalized polymers to prepare the adhesive.[3,4] An interesting approach with functionalized polymers toward the biomedical use of adhesives involved formation of multi-component hydrogels.[5] Some of the examples are development of a bioadhesive gel by coupling the polysaccharide alginate and DOPA for treatment of atherosclerotic plaques in a mouse model,[6] hydrogel formed by mixing recombinant mussel adhesive proteins and the polysaccharide hyaluronic acid to be used as a self-adhesive micro-encapsulated drug carriers,[7] hydrogel made from citric acid, polyethylene glycol, and DOPA used for healing of incision wound,[8] etc.

In the biological world, the adhesive mucus released by gastropod snails and slugs represents yet another kind of multicomponent hydrogel adhesive. These organisms secrete mucus in order to carry out a variety of functions such as locomotion or attachment on horizontal and vertical surfaces, defense against predators, protection from a dynamic (wave swept or tidal) environment, or desiccation and locating prey item and mate.[9−11] Many of these functions are based on the ability of the mucus to act as an adhesive, especially when it is more viscous and stiffer than the usual trail mucus. This special thicker and stickier mucus, termed as adhesive mucus, is believed to provide stronger attachment to a substrate than the trail mucus does.[9−13] It is possible to collect this mucus after secretion and use it as a hydrogel adhesive. However, any practical use of this adhesive has not been sufficiently explored.

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Unlike many other biological adhesives, studies on gastropod mucus were not restricted to its structural or mechanical properties but had also been explored for its medical and pharmaceutical use. Antimicrobial activities were detected in the mucus of the snails *Cornu aspersum*,[14] *Helix aspersa*,[15] and *Achatina fulica*.[16] Mucus from different slugs and snails were found to facilitate wound healing and prevent infection.[17−19] Also, Gentili *et al.* showed the ability of the *H. aspersa* mucus to provide protection against O_3-induced oxidative damage.[20] Given all these findings, it is extremely likely that we can directly obtain an adhesive hydrogel with an added therapeutic value from these invertebrates.

Irrespective of their biological use, trail and adhesive mucus from different gastropods are known to have similar biochemical compositions, consisting of polysaccharides, proteins, and metals.[9,10,12,21] Smith and his group carried out biochemical studies elucidating the roles of proteins and metals in the gel forming reactions.[12,21−25] Most of these studies are based on the slug *Arion subfuscus*. It was reported that its mucus contains considerable amounts of different metals, such as iron (Fe), calcium (Ca), magnesium (Mg), zinc (Zn), etc., which are responsible for the cross-linking of the polymers.[21,25] It was shown that principal cross-linking reactions are the direct divalent metal (Cu, Ca, and Mg)-based cross-links, where metals interact with negatively charged chemical groups and help bringing the polymers together. In addition, some proteins undergo Fe-dependent oxidation and form imine bonds facilitating cross-linking. Wilks *et al.* described that the interpenetrating network structure of this multicomponent hydrogel is responsible for its extraordinary strength.[26] These studies inspired manufacturing of a biomimetic tissue adhesive with a similar interpenetrating network.[27]

In spite of the detailed structural and biochemical studies, there was no systematic report on the adhesive function of the original slug mucus. Therefore, we focused on elucidation of the adhesive function of gastropod mucus. Our earlier work was on the mucus from the snail *Macrochlamys indica*, and we reported its adhesion on wet surfaces.[28] Although this snail mucus could attach on wet surfaces, it lacked stability under water, in which its lifetime was only a couple of hours. In fact, the natural habitat of gastropods is not aquatic, so any underwater adhesive function of their mucus was neither expected nor known. Consequently, any possible use as biomedical adhesive was less anticipated. However, we envisioned that if a natural habitat of some gastropods is closer to water bodies, then their act of using the adhesive mucus would be more adapted to the presence of water. Our quest for such adhesives resulted in our collection of the slug *Laevicaulis alte* from humid regions, which endure lots of rain throughout the year. Preliminary observation of this slug mucus showed stronger force of attachment and greater stability under water than other gastropod mucus that we had known yet was weaker than reported biomimetic adhesives.

Based on the knowledge that metals are the main cross-linking components in the hydrogel, we hypothesized that the hydrogel can be strengthened by external addition of metals. Accordingly, the likelihood of enhancement of adhesive strength was explored by simple modifications, treatments with salts of four different metals: calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), cupric chloride ($CuCl_2$), and zinc chloride ($ZnCl_2$). In this paper, the effects of all four metals on the underwater stability and adhesive property of the hydrogel have been compared. We hope our investigation shows promise of availing an instant hydrogel adhesive strong enough for biomedical uses and also generate new inspirations and ideas for designing underwater adhesives.

RESULTS

Adhesive mucus was collected from slugs in DW (untreated) or in one of the four metal salt solutions in DW (deionized water)—$CaCl_2$, $MgCl_2$, $CuCl_2$, and $ZnCl_2$ (metal treated)—and differential effects of these metals on the underwater adhesive property and stability of the mucus in water were studied.

Analysis of Intrinsic Metal Content and Treatment with Excess Metal. Intrinsic metal content of the slug mucus was determined by AAS (atomic absorption spectroscopy) analysis. This method was used to detect the amounts of Ca, Mg, Cu, and Zn, which are generally found in the slug mucus. Mucus from three slugs were mixed and used for each analysis. Average values for each metal came from three such analyses. As shown in Figure 1, average amounts of Ca and Mg per

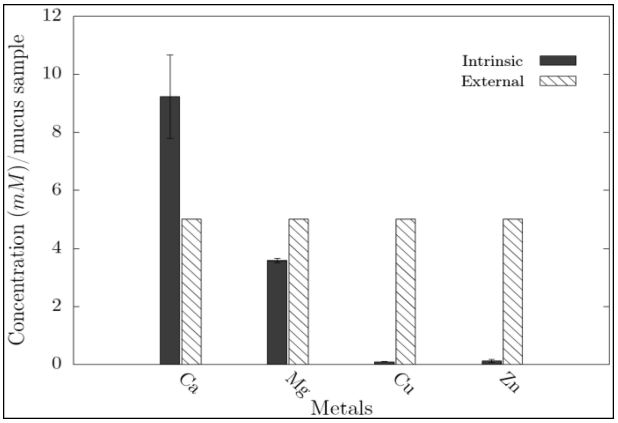

Figure 1. Relative amounts of intrinsic and the externally added metals in slug mucus. Each bar corresponding to an intrinsic metal content (box, closed) represents the average of three estimations; the error bar represents the standard deviation. Each bar corresponding to externally added metal (box, open) represents the calculated amount of the added metal.

mucus sample are much higher than those of Cu and Zn. One of the factors behind this difference may be the relative abundance of these metals in the habitat of these snails.

In order to study the effect of each metal on the mucus, it was treated with 5 mM chloride salt solutions of the particular metal ($CaCl_2$, $MgCl_2$, $CuCl_2$, or $ZnCl_2$) as described in the Materials and Methods. The mucus was exposed to the external metal during its release and subsequent cross-linking to a hydrogel so that the external metal could affect the cross-linking process. However, the intrinsic metals interacted with the polymer components even before the cross-linking started. Therefore, external metals might not have exactly the same effect as the intrinsic metals. Considering this possibility, we aimed to keep the amount of all external metals the same to make a systematic comparison between their effects. Immediately after the hydrogel formation, the metal-treated and untreated mucus samples were subjected to adhesive studies.

Figure 1 shows the comparison between the intrinsic metal content and the amount of the externally added metals. The intrinsic Ca content was already high in the mucus; therefore,

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the externally added Ca was a small addition to the existing amount. In the case of Mg, the relative amount of the added metal was close to the intrinsic content, while in the case of the transition metals, the external additions were significantly higher than the intrinsic amount. Irrespective of treatment, all mucus contained its intrinsic amounts of Ca (avg. 9.2 mM), Mg (avg. 3.6 mM), Cu (avg. 0.1 mM), and Zn (avg. 0.1 mM).

Underwater Attachment of Surfaces with Mucus Adhesive—Force and Duration of Attachment. Untreated and metal-treated mucus were used as adhesives to attach glass slides by applying light pressure. The attached slides were then immersed under water (see Supporting Information video, Movie S1). Our aim was to know the longest time the adhesive could keep the slides attached under water and to measure the force of attachment. For this purpose, at intervals of 1−3 days, pairs of attached slides were taken out of water, and the adhesive force was measured by Method 2 (see Materials and Methods Section).

As shown in Figure 2, for each metal treatment, the force gradually decreased with increasing the number of days of incubation under water. The longest time for which the slides remained attached varied with the type of initial treatment of the mucus, in the order Cu^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+} > DW. Slides with the Cu^{2+}-treated gel remained attached for about 18−22 days, which was the longest among all five conditions. These gels also showed the highest average adhesive force, approximately 18 kPa after 24 h in water. Zinc-treated mucus could keep the slides attached under water for 15−18 days, where the average force was 17 kPa after 24 h in water. Slides with the Mg^{2+}-treated gel remained attached for about 8−9 days, with an average force of 15 kPa after 24 h, while Ca^{2+}-treated gels could keep the slides attached for only 5−6 days. The untreated mucus also could keep the slides attached for about 5−6 days and showed an average adhesive force of 5 kPa after 24 h under water. Thus, it was observed that metal treatment of mucus improved both the stability and the strength of the underwater attachment of glass surfaces. Dependence of the strength and duration of attachment of surfaces on the metal treatment followed the trend established by the Irving−Williams series, in the order Ca^{2+} < Mg^{2+} < Cu^{2+} > Zn^{2+}.[29]

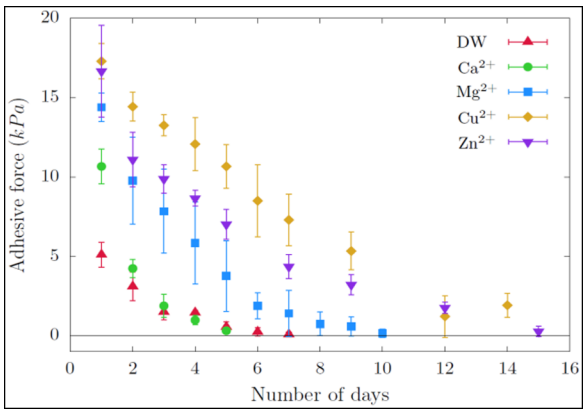

Figure 2. Underwater adhesive property of slug mucus. Adhesive force needed to detach slides that were attached with slug adhesive mucus and kept under water for several days. The adhesive mucus, used for this purpose, was untreated (collected in DW) or treated with Cu^{2+}, Zn^{2+}, Mg^{2+}, or Ca^{2+}. Each data point represents an average of four measurements; the error bar represents standard deviation.

The ability to keep surfaces attached for days under water suitably defined its adhesive function and was an effective way to compare between the enhancing effects of different metals. The higher duration (8 to 20 days) achieved was supportive of its probable application in the biomedical field. It should be noted that the mucus had limited exposure to water through a gap of about 100 μm between the two surfaces. It may be the most common scenario encountered as an underwater adhesive, but from the practical point of view, the possibility of greater exposure to water cannot be ignored. More exposure could increase the possibility of faster destabilization of the hydrogel and required additional experiments to check its stability toward greater interaction with water. These experiments have been described in the following "Effect of Water Exposure on the Integrity of the Mucus Gel" Section.

Probe Tack Test and Generation of the Adhesive Stress vs Strain Curve of the Mucus. Untreated and metal-treated mucus samples were subjected to the probe tack test using Method 1, with a combination of a motorized stage and a load cell sensor (Figure 3C), as described in Materials and Methods. For this test, the mucus samples were used to attach two glass slides for a fixed contact time of 10 min followed by the measurement of the pull-off force. These resulted in the adhesive force vs distance graphs for each sample.

Figure 4 shows the representative adhesive stress vs strain curve, which was obtained with the Mg^{2+}-treated mucus. Each data point represents an average of four measurements, and the corresponding error bar represents the standard deviation. The zero point on the x axis in the graph indicates the moment when the slides remained attached with the adhesive in between with zero force. As the instrument pulled apart the slides at a constant rate, the force of separation was recorded against the distance of separation of the slides. Adhesive stress was calculated by dividing the force with the area of the adhesive layer between the two surfaces. Strain was calculated by dividing the distance of separation with the initial thickness of the adhesive layer. The resulting adhesive stress vs strain curve recorded a rise in stress with increase in strain. After reaching a maximum, the stress decreased. Finally, the adhesive yielded and the stress came down to zero.

The graph in Figure 4 showed a slower decrease toward the end of the sharp fall, creating a shoulder. Before complete separation, the graph continued with extended strain creating a plateau. As explained by Deplace *et al.*, the slow decrease and the plateau in the graph is the result of deformation in the gel.[30] The deformation was visible in our experiments as the stretching of the mucus during pulling the slides apart. The deformation relieved the stress produced in an attempt to separate the surfaces.

Effect of Water Exposure on the Integrity of the Mucus Gel. As an underwater adhesive, the mucus is likely to be often immersed in water, and the extensive contact with water may affect the integrity of the hydrogel structure and its ability to function as an adhesive. Therefore, in the present section, we investigate the stability of the mucus gel under prolonged water exposure. For this, untreated and metal-treated mucus samples were kept immersed under water for different time periods. Integrity was checked by physical observation of the wet mucus and estimated the loss of protein and carbohydrate components due to water exposure.

It was found that, as the mucus samples were kept immersed in water for 3−5 days, there was a visible decrease in firmness of the untreated and the Ca^{2+} and Mg^{2+}-treated samples. These

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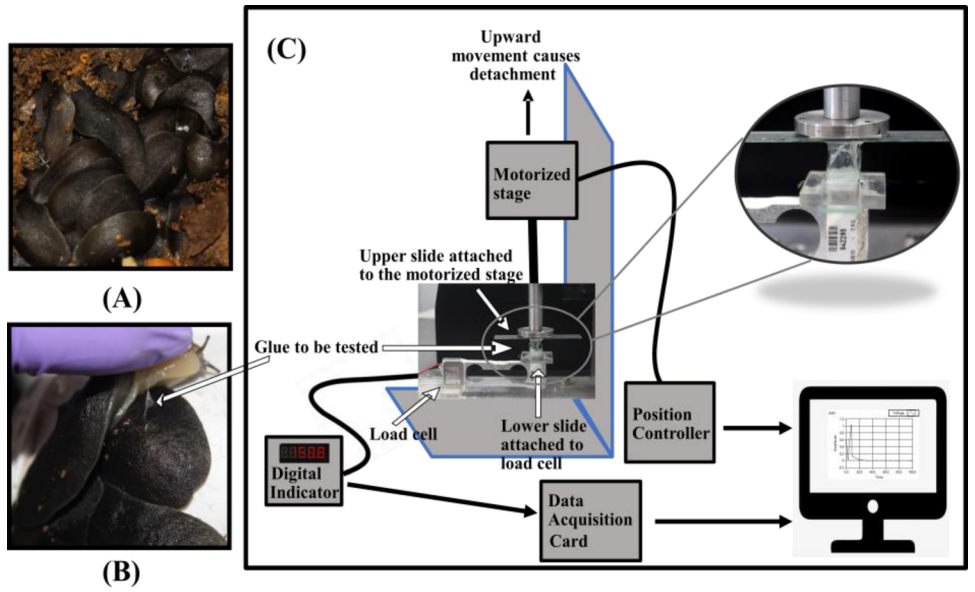

Figure 3. Slug adhesive mucus and measurement of its adhesive property (Method 1). (A) Young slugs clumped with each other in soil. (B) Slugs sticking to each other with adhesive mucus. (C) Design of the load cell—motorized stage assembly for the measurement of adhesive force. This system was used to measure the force required to detach slides that were attached with the slug adhesive mucus.

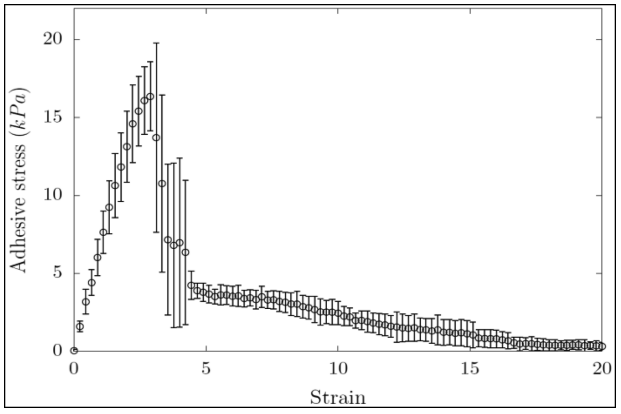

Figure 4. Representative adhesive stress vs strain curves of the mucus. Adhesive stress vs strain curve of Mg^{2+}-treated mucus, obtained from a set of four tests with mucus samples collected from four individual slugs. Each data point is an average of four stress values at a particular strain; the error bar represents standard deviation.

samples continued to remain in the dilute gel form until 24 h after which they got dissolved in water. Mucus samples treated with Cu^{2+} and Zn^{2+} lost their stiffness relatively slowly and remained in their gel form beyond 72 h.

At different time points, samples were withdrawn from the aqueous media that was used to keep each mucus sample immersed, and the loss of proteins and carbohydrates was estimated. As shown in Figure 5A,B, at the beginning (0 h water exposure), there was no significant difference between the untreated and the metal-treated samples in terms of loss of proteins and carbohydrates. After 2 h [Figure 5A], the loss of proteins increased in all samples. At a 24 h time point, the loss was significantly higher both in Ca^{2+} and Mg^{2+}-treated samples than in untreated and Cu^{2+} and Zn^{2+}-treated mucus samples.

There was very little loss of carbohydrates at the 0 and 2 h time points [Figure 5B] for all samples. After 2 h, it increased considerably in the untreated and Ca^{2+} and Mg^{2+}-treated mucus but not in the Cu^{2+} and Zn^{2+}-treated ones. At 12 h, the concentrations of carbohydrates in the surrounding aqueous media of untreated and Mg^{2+}-treated samples reached maximum, around 700 μg/mL, which was significantly higher than other samples. The loss of carbohydrates from the Ca^{2+}-treated sample was slower than the untreated and the Mg^{2+}-treated mucus and reached a similarly high value at 24 h. After reaching the maximum, the concentrations in the case of untreated and the Ca^{2+} and Mg^{2+}-treated samples subsequently decreased, which could be a result of the action of carbohydrate degrading enzymes (unpublished result) that are present in the mucus and could degrade the already released carbohydrate. Since, after 24 h, the untreated and the Ca^{2+} and Mg^{2+}-treated samples practically lost the gel structure and got dissolved in the surrounding media, withdrawal of samples and estimation of proteins and carbohydrates were stopped. In the case of Cu^{2+} and Zn^{2+}-treated samples, estimation was carried out until 72 h. The loss of protein and carbohydrate was found to increase steadily but at a slower rate than the untreated and Ca^{2+} and Mg^{2+}-treated samples. These observations demonstrated that the integrity of the untreated and the Ca^{2+} and Mg^{2+}-treated samples weakened faster than Cu^{2+} and Zn^{2+}-treated samples.

The maximum stability (72 h) of mucus found in this experiment appears to be less than the duration (3−20 days) of attachment of surfaces shown in the experimental section "Underwater Attachment of Surfaces with Mucus Adhesive" and associated figure (Figure 2). This was expected because of the difference of exposure to water in these two experiments; the mucus was sandwiched between glass surfaces in the case of Figure 2, while it was fully immersed in water in the present case.

Effect of Metal Treatment and Water Exposure on the Adhesion Energy of the Mucus. The effect of prolonged water exposure on adhesive properties were studied by keeping mucus samples immersed in water for time periods 0, 2, 12, 24, 48, and 72 h and then subjecting them to the probe tack test. Adhesion energy (w) was obtained by

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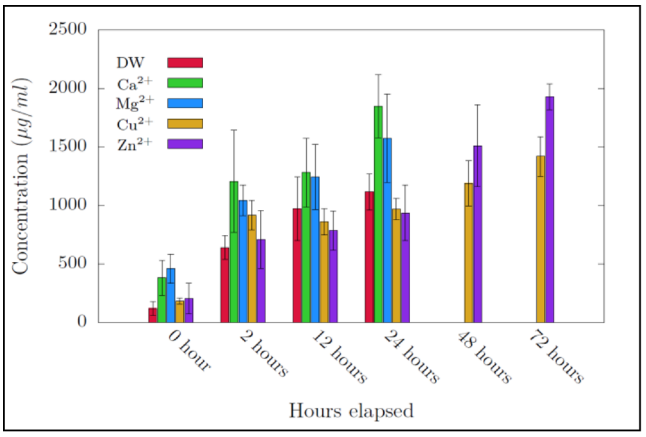
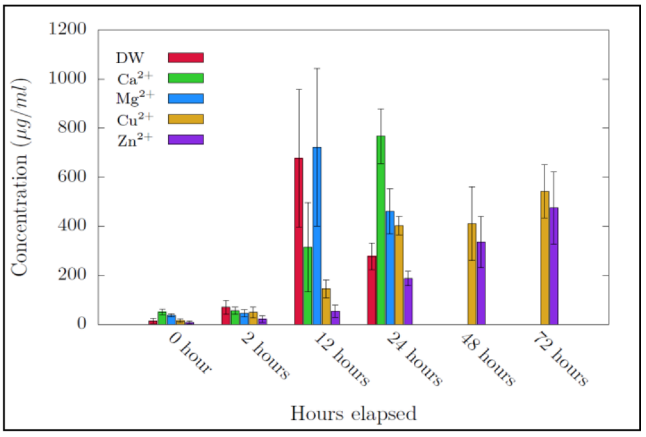


(A)



(B)

Figure 5. Loss of proteins and carbohydrates from mucus samples in the surrounding water. Bar graph showing the average amounts of (A) proteins and (B) carbohydrates released from mucus hydrogel samples that were kept immersed in water for different durations. Each bar represents the average of four measurements; the error bar represents the standard deviation.

integrating the corresponding adhesive stress vs strain curve and using the following formula

$$w = d \int \sigma \, d\varepsilon$$

where d represents the sample thickness, σ represents the adhesive stress, and ε represents the strain.[31] The results for adhesion energy are shown in Figure 6 for all the studied conditions. Initially, for 0 h water exposure, the adhesion energy of the untreated mucus was approx. 18 J/m^2, which was not significantly different from the values in any of the metal-treated mucus. After 2 h of water exposure, this value increased up to 25 J/m^2, which was significantly higher than those of metal-treated mucus. During this time period, adhesion energy of none of the metal-treated mucus changed significantly. These results of 0 and 2 h water exposure were in contrast to the underwater attachment results shown in Figure 2, where all metal-treated mucus showed greater adhesive force than the untreated mucus. This is perhaps due to the difference in contact times in these two experiments. In the present experiment, the glass slides were attached for 10 min. The increase in stiffness by metal treatment may have caused the

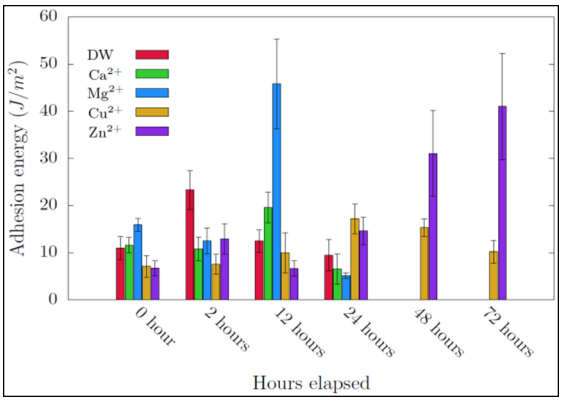


Figure 6. Adhesion energy of mucus samples after water exposure. Bar graph showing the adhesion energy of mucus hydrogel samples that were kept immersed in water for different durations. Each bar represents the average of four estimations; the error bar represents the standard deviation.

mucus form less contact with the glass surface in this short time. On the other hand, in the experiment corresponding to Figure 2, the glass surfaces were attached under water for 24 h and more, giving the adhesive enough time to form as many adhesive bonds as possible.[32]

With extended water exposure, the adhesion energy decreased in untreated mucus but increased in the metal-treated ones. As shown in Figure 5B, there was a sudden increase in the loss of carbohydrates in the time range of 2−12 h. This may have decreased the compactness with a resultant decrease in stiffness of the mucus, enabling it to make better contact with the surface. The increase in adhesion energy was more substantial in Ca^{2+} or Mg^{2+}-treated mucus than in the Cu^{2+} and Zn^{2+}-treated mucus. At 12 h, the adhesion energy reached a maximum average value of 46 J/m^2 in the case of Mg^{2+}-treated samples, and 20 J/m^2 in the case of Ca^{2+}-treated samples. On further increasing the time to 24 h, the adhesion energy decreased for the untreated and Ca^{2+} or Mg^{2+}-treated samples. Beyond 24 h, these treated mucus, as well as untreated ones, started getting dissolved. Our understanding is that this is due to a combined effect of water related weakening of the cross-linking force and general enzyme related biodegradation of the polymer molecules. Thus, although treatment with Mg^{2+} could increase the adhesive strength of the mucus, it could not increase its stability.

In mucus treated with transition metals, Cu^{2+} and Zn^{2+}, increase in adhesion energy was more gradual. As Cu^{2+} and Zn^{2+}-treated gels remained intact at least until 72 h, probe tack tests were carried out till 72 h. In the case of Zn^{2+} treated mucus, adhesion energy increased beyond 24 h, with a maximum average value of 41 J/m^2 at 72 h. This was comparable to the maximum average value of the Mg^{2+}-treated sample at 12 h. Less variation was observed in the case of Cu^{2+}-treated samples, where adhesion energy increased up to 17 J/m^2 at 24 h and then decreased gradually. Therefore, the slower loss of integrity of Cu^{2+} and Zn^{2+}-treated mucus was reflected in the slower increase in adhesion energy compared to those of Ca^{2+} and Mg^{2+}-treated mucus. Overall, these results showed that, while all untreated or metal-treated mucus hydrogels underwent a gradual loss of integrity in the presence of water, the treatment with Cu^{2+} and Zn^{2+} could, in fact, increase the underwater stability of the hydrogel.

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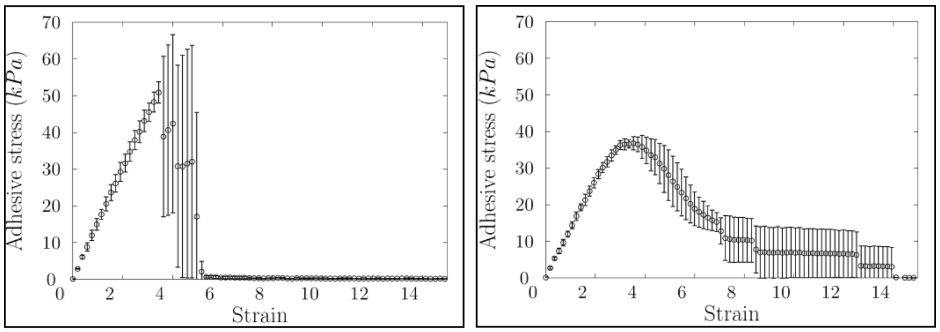

Figure 7. Adhesive stress vs strain curves associated with the maximum adhesion energy of the Mg^{2+} and Zn^{2+}-treated mucus. Adhesive stress vs strain curves of (A) Mg^{2+}-treated mucus at 12 h water exposure and (B) Zn^{2+}-treated mucus at 72 h water exposure. Each curve was obtained from a set of four tests with mucus samples collected from four individual slugs. Each data point is an average of four stress values at a particular strain; the error bar represents standard deviation.

Underwater stability was not the only aspect where the effects of Ca^{2+} or Mg^{2+} treatment on the adhesive differed from the effects of Cu^{2+} or Zn^{2+} treatment. There were also differences in the deformability and cohesive properties of the gel. Variances were observed in the shapes of the stress vs strain curves based on the metal treatments. Ca^{2+} and Mg^{2+}-treated mucus generally showed higher peak adhesive stress followed by a sharper decrease in stress than other mucus, while Cu^{2+} and Zn^{2+}-treated mucus showed lower peak adhesive stress followed by a slower decrease in stress. Figure 7A,B shows the stress vs strain curves corresponding to the maximum average adhesion energy values of the Mg^{2+}-treated mucus (at 12 h of water exposure) and Zn^{2+}-treated mucus (at 72 h of water exposure), respectively. The curve corresponding to Mg^{2+}-treated mucus showed a sharp peak with an average stress value of 60 kPa followed by a rapid fall, while the curve corresponding to the Zn^{2+}-treated mucus showed an average peak stress value of 38 kPa, with a gradual decrease and plateau formation, indicating greater deformability than the Mg^{2+}-treated mucus. Thus, although Mg^{2+}-treated and Zn^{2+}-treated hydrogels could achieve closely similar values of energy of adhesion, their behavior as underwater adhesives was different. Also, it was noted that, in the case of Cu^{2+} and Zn^{2+} treatments, separation of the slides caused adhesive separation with the entire gel remaining in one of the two detached surfaces, indicating the cohesive nature of the gel. On the other hand, in the cases of untreated and Mg^{2+} and Ca^{2+}-treated mucus, separation of the slides caused cohesive failure with part of the gel remaining in each of the two detached surfaces. The adhesive separation of the Cu^{2+} and Zn^{2+}-treated gels was indicative of a cohesive interaction stronger than hydrogel glass interfacial interaction. Formation of small air pockets at the hydrogel glass interface[31] could have been faster in these cases than in other gels, causing less peak adhesive force.

Throughout the 72 h time period, the Cu^{2+}-treated hydrogel was visibly the most intact and showed a slower rate of loss of protein and carbohydrate components. However, notwithstanding this, the maximum adhesion energy of Cu^{2+}-treated hydrogel was one of the lowest among all the untreated and metal-treated hydrogels. Although treatment with Cu^{2+} increased the cohesive interaction, it negatively affected the interaction of the hydrogel with the glass surface for short contact time such as 10 min. By comparison of the enhancing effects of all four metals on the underwater adhesive property of the mucus, the effect of Zn^{2+} was considered the best since treatment with Zn^{2+} could increase the stability of the gel under water and could also increase the adhesive strength.

DISCUSSION

In this work, the underwater adhesive property of the adhesive mucus of the slug *L. alte* has been investigated. Also, the possibility of upgrading the adhesive property by the external addition of metals has been explored.

There are inadequate examples of adhesive studies on isolated biological underwater adhesives, probably because of their rapid curing before collection and their inherent sample to sample variability. Hence, there was little scope of comparing our results with the performance of other underwater adhesives. In the case of slug adhesive mucus, the semisolid hydrogel structure made it possible to use it as an adhesive after its collection. For each experiment, adhesive studies were carried out multiple times with different batches of slugs collected at different times of the year. Thus, multiple repetitions established a general range of values of the adhesive force of this biological hydrogel.

Mucus from invertebrates is mainly useful for temporary attachment in a wet environment. As the organisms release their mucus, it may be designed to undergo modification by specific environmental components. If the mucus is used for attachment on water-immersed surfaces, there may be greater chances of modification by the metals dissolved in water. Example of such an environmental effect could be seen in the effect of sea water on marine adhesives such as those from mussels or sandcastle worms.[33]

Metals have been found to play an important role in the adhesives from a variety of aquatic organisms. For example, self-assembly of collagen-based copolymers in mussel byssus through metal binding by histidine-rich sequences,[34] the pH-triggered interaction of phosphoproteins with Ca^{2+} or Mg^{2+} in sandcastle glue of *Phragmatopoma*,[35] interaction of Ca^{2+} with phosphorylated serine in caddisfly larvae silk proteins,[36] iron–DOPA complexes in sandcastle glue, and mussel adhesive plaques.[1] However, enough information on the effect of transition metals like Cu^{2+} or Zn^{2+} on underwater adhesion is lacking. In our study, we observed that the general effect of the transition metals on the adhesive was different from the effects of Ca^{2+} and Mg^{2+}. Cu^{2+} and Zn^{2+} specifically caused a considerable increase in the stability of the gel toward

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prolonged water exposure. Additionally, treatment with Cu^{2+} and Zn^{2+} reduced the rigid nature of the mucus, made it more cohesive, and increased the stretchability of the mucus gel. As discussed by earlier researchers, the merit of transition metals, like Cu^{2+} and Zn^{2+}, lies in their extra ability to form coordinate covalent bonds, which is more effective than electrostatic interaction in providing protection against water-related weakening of bonds.[37] Thus, a special stabilizing effect of transition metals observed in our work can be considered due, at least in part, to the formation of coordinate covalent bonds with histidines present in the proteins.[1] In this context, it can be mentioned that a recent study of analyzing sequences of the mucus proteins of the slug *A. subfuscus* detected histidine-rich motifs, which are generally known to bind Cu^{2+} and Zn^{2+}.[38] Also, an abundance of Zn^{2+} was found in the same mucus[25] but so far no structural or mechanical effect of such interaction (between this Zn^{2+} and the Zn^{2+} binding domain) was shown. Although similar studies to identify mucus proteins are yet to be done for the present slug, our results suggest that such Zn^{2+}-dependent interaction occurred in its mucus, providing structural stability in the presence of water.

Although gastropod adhesive mucus has its own intrinsic metal content, the results of the present work could be related mainly to the amounts of the externally added metals. For example, there is a large difference between the intrinsic amounts of Mg^{2+} and Zn^{2+} in the mucus. Nevertheless, the maximum adhesion energies of Mg^{2+} and Zn^{2+}-treated mucus were almost the same, which could be explained by the same external amount (Figure 6). During the experiment corresponding to surfaces attached with long contact time (Figure 2), the improvement by metal treatment followed the order $Ca^{2+} < Mg^{2+} < Cu^{2+} > Zn^{2+}$, the same trend established by the Irving−Williams series of stability indices of metal complexes. According to this series, Ca^{2+}-dependent complex shows the lowest stability among all the metals. This may be the reason why the performance of the Ca^{2+}-treated mucus was almost the same as that of the untreated one. During the probe tack test corresponding to a 10 min contact time (Figures 5 and 6), the stabilities of the metal-treated hydrogels followed almost the same series. However, the same order was not directly reflected in the adhesive property. In spite of the fact that the Cu^{2+}-treated gels were the most stable and intact, its adhesion on the glass surface was not as effective as other mucus samples. We postulate that the higher integrity prevented it from establishing strong enough interfacial contact in the short time period of 10 min. From these results, it can be concluded that if this gel is to be used as the wet adhesive and a fast effect is needed, then it should be treated with Zn^{2+} for stronger adhesion.

Temporary hydrogel adhesives may find their biomedical applications in topical wound healing, mucoadhesive drug delivery systems, or even as tissue adhesives. The slug mucus gel provides an example of a multicomponent hydrogel with a promised flexibility of multipurpose use. With a demonstration of the ability to keep two surfaces attached for a minimum of 5 days in the case of untreated mucus to a maximum of 20 days in the case of metal-treated ones, this study points toward the potential of developing mucus-based hydrogels for biomedical use.

CONCLUSIONS

Adhesive mucus from the garden slug *Laevicaulis alte* could be used as a temporary hydrogel-based underwater adhesive. Both the time and force of adhesion could be improved to a different extent by metal treatment using the salts, $CuCl_2$, $ZnCl_2$, $MgCl_2$, or $CaCl_2$. Treatment with the transition metals, Cu^{2+} and Zn^{2+}, could increase the stability of the mucus against exposure to water. This study presents a novel method of external modification of gastropod mucus adhesives. It shows the unique ability of the mucus to be used as an effective adhesive in wet conditions, along with the flexibility of undergoing easy modifications. Such properties can make it suitable for hydrogel-based biomedical applications. This multicomponent hydrogel may present the flexibility of modification of each component separately to achieve a multifaceted character suitable for various types of uses.

MATERIALS AND METHODS

Materials. All the reagents, including metal salts, were bought from HiMedia Laboratories, Mumbai, India. Microscope glass slides were used for adhesive experiments. The deionized water (DW) used for all the experiments was MilliQ water.

Collection and Maintenance of Slugs. Slugs of the species *Laevicaulis alte* [Figure 3A,B] were collected from the eastern part of India. They were maintained in the laboratory for about 2 weeks after which they were returned to the wilderness. They were kept in soil in earthenware pots and fed on vegetables. Water was sprinkled once or twice a day to keep the soil moist and the environment humid. The juvenile slugs clumped with each other with the help of a sticky mucus. This mucus was collected for the purpose of the present study.

Collection of Slug Mucus and Metal Treatment. Slugs were taken out of the soil, washed with distilled water, dried with paper towels, and were placed on glass slides.[28] A small volume (250 μL) of deionized water (DW) was added to the back of the slug, which was gently scraped with the help of a spatula to induce mucus secretion. The mucus mixed with the water as it was being secreted and immediately formed a semisolid hydrogel. The hydrogel was stored on ice for further study on the same day, and the slug was transferred back to its habitat. The mucus, thus collected in DW, was called the untreated mucus. During collection, if DW was replaced with metal salt solutions such as $CaCl_2$, $MgCl_2$, $CuCl_2$, or $ZnCl_2$, each of 5 mM in concentration, the mucus was considered treated with the respective metal and was called the metal-treated mucus.

Detection of Intrinsic Metal Content in the Slug Mucus. Mucus samples from three slugs were collected, each in 500 μL DW by the process described above. For metal detection, these samples were pooled together, 1.5 mL of DW was added to it, and incubated for 2 h. The samples were then sonicated for 2 min at 60% amplitude and a 10 s pulse rate with an ultrasonicator model VCX130 (with probe microtip of diameter of 3 mm) from Sonics & Materials Inc. Newtown, CT, USA, and centrifuged at 13000 rpm for 15 min. The supernatant was then collected (no pellet observed) and acid digested in 50 mL of aqua regia. This extract was then subjected to atomic absorption spectroscopic (AAS) (AA-6300 Shimadzu) analysis for detection of calcium, magnesium, copper, and zinc. The whole process was carried out in triplicates.

Underwater Attachment of Surfaces with Mucus Adhesive. Untreated and metal-treated mucus were used as adhesive to attach glass slides, which were immersed under water. At intervals of 1−3 days, pairs of slides were taken out of the water, and the detachment force was measured using

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Method 2 for measurement of adhesive force (see the section Measurement of Adhesive Force). This process was continued until the slides were detached automatically.

Water Exposure of the Mucus Gel and Checking Its Integrity. Untreated and metal-treated mucus samples were kept immersed in 1 mL of DW. The surrounding water (5 μL) was collected at different time points (0, 2, 12, 24, 48, and 72 h) and was subjected to the protein and carbohydrate estimation. Fresh 5 μL of water was added back after each collection. Carbohydrate estimation was done by the orcinol–sulfuric acid method and protein estimation by the bicinchoninic acid assay.

Water Exposure of the Mucus Gel and Studying Its Adhesive Property. Untreated and metal-treated mucus samples were kept immersed in 1 mL of DW for different time periods, 0, 2, 12, 24, 48, and 72 h. The wet mucus was removed from water, the water meniscus at its edges was removed with tissue paper, and then the solution was subjected to the probe tack test using Method 1 of Measurement of Adhesive Force Section.

Measurement of Adhesive Force of the Wet Mucus Gel. *Adhesive Property Was Measured by a Pull-off Test by Two Different Methods.* Method 1: A layer of the mucus gel was sandwiched between two glass slides. The slides were then separated at a controlled rate by applying a pull-off load, generating the adhesive force vs distance curve. The experimental arrangement is schematically described in Figure 3C, in which the lower slide was attached to a load cell (Eltek Systems, Mumbai, India), interfaced with a data acquisition card and computer. The upper slide was connected to a motorized translational stage (Holmarc Opti-Mechatronics Pvt. Ltd., Kochi, India), which could be moved at a controlled rate aided by position controller software. The position and movement of the stage was recorded by the software, which assisted in the calculation of the distance between the upper and the lower slides. The gel was kept on the lower slide, while the upper slide was moved downward with the help of the motorized stage and brought into contact with the gel. The motor was stopped when a contact force of 1.47 N (corresponding to a load of 150 g) was reached. Our preliminary studies showed that a minimum contact force of 1.47 N was needed for the upper slide to establish proper contact with the gel. The adhesive remained sandwiched between the slides placed in a cross position. It covered an overlapping area of 1 cm^2.

During application of pressure to the slides, it was visible that the hydrogel spread uniformly on the overlapping region. Slides were kept compressed for 10 min to allow relaxation of the gel. During this time, the load cell sensor attached with the slides detected the adjustment of stress. Furthermore, each experiment was repeated multiple times to even out any possible error of nonuniformity.

After 10 min of contact, the upper slide was pulled upward by the motorized stage at a controlled rate, causing the adhesive to stretch between the two glass slides. Eventually, the adhesive yielded and the slides were detached. The strain exerted on the adhesive, as the distance between the two slides increased, was converted to the corresponding adhesive stress vs strain curves.

Method 2: An alternative method was used in experiments where glass slides were attached with mucus gel with hours of contact time, after which the pull off force needed to detach the slides (adhesive force) was measured. In this case, the force was measured with a locally made instrument.[28] In this method, the attached glass slides were placed on a small platform. A weighing pan was attached through a pulley to the upper slide, while the lower slide remained fixed to the platform (Figure S1, Supporting Information). Increasing weights were placed on the weighing pan till the attachment failed. Adhesive force in Pascal (Pa = N/m^2) was manually calculated as the maximum weight needed to detach the glass slides per unit of overlapping area.

When the slides were attached for hours, the measurement of the adhesive force could not be carried out by Method 1. Method 1 required attaching each glass slide separately, one with the load cell and the other with the motorized stage. This was found to be technically difficult since both the slides were already attached to each other with slug adhesive.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06132.

(Figure S1) Measurement of adhesive force by Method 2 (PDF)

(Movie S1) Video showing the attachment of two glass surfaces under water using slug mucus (MP4)

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Author Contributions

J.N. and S.V. were both responsible for the experiments. J.N. carried out most of the mechanical measurements, helped refine the methods, and analyzed the data. S.V. carried out biochemical estimations of the mucus components. A.G. proposed the research idea, was responsible for designing of experiments and interpretation of results, and drafting and finalization of the article.

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Notes

The authors declare no competing financial interest.

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assembly of instruments for adhesion experiments and his valuable comments on the data analysis.

ABBREVIATIONS

AAS	atomic absorption spectroscopy
Ca	calcium
$CaCl_2$	calcium chloride
Cu	copper
$CuCl_2$	copper chloride
DW	deionized water
Fe	iron
kPa	kiloPascal
Mg	magnesium
$MgCl_2$	magnesium chloride
Zn	zinc
$ZnCl_2$	zinc chloride

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