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Bi-functional Roles of Ca-Y Zeolite in Treatment of Ethanol-HCl Induced Gastric Ulcer in a Mice Model†

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Calcium (II) exchanged zeolite Y (Ca-Y) was prepared and evaluated with ethanol-HCl induced gastric ulcer in a mice model. Beneficial from the high procoagulant activity with good resistance to gastric juice and anti-acid capability of Ca-Y, a significantly reduced ulcer area percentage from 35.1 ± 4.4% to 11.5 ± 1.9% was achieved at an oral dosage of 5.0 g/kg, along with increased intragastric PH from 2.0 ± 0.5 to 4.5 ± 0.5.

Peptic ulcer include gastric and duodenal ulcer, is one of the major human pathologies that affects 10-15% of the world population.[1] It is a heterogeneous disease of multifactorial etiology, resulting from consumption of nonsteroidal anti-inflammatory drugs (NSAIDs), infection of *Helicobacter pylori*, excessive alcohol intake as well as irregular dietary manner and suffering from severe stress.[2] The direct etiological factor is the disorder of balance between defensive factors (mucus and surface epithelial cell) and offensive factors (hydrochloric acid and pepsin).[3] The imbalance of this gastrointestinal self-protective system can cause mucosal damage, which may develop into gastric ulcer, sometimes with consequent complications like bleeding, perforation and gastric outlet obstruction.[4] On account of adverse environment (hydrochloric acid, pepsin, and chyme), the process of hemostasis and tissue repair inside stomach would face many more obstacles compared to external wound healing. Therefore additional therapies, with hemostatic like glue or clotting products are being more frequently used in endoscopic therapy of gastrointestinal bleeding.[5]

Mineral based hemostatic, such as zeolite and clay, has been widely proved to be effective for controlling external hemorrhage in the last decade.[6, 7] Recently, it has also been demonstrated to give promising results for therapy of gastric diseases in several animal and clinical trials.[8,9] While the exact mechanisms of action remains unclear, the excellent performances are generally attributed to the special physical and chemical properties of these porous materials. Barkun *et al* reported that the *Hemospray*™ powder demonstrates adhesive properties. It acts as a bandage coupled with local tamponade effect upon contacting with moisture, and dehydrates tissue through absorption of water, which concentrates clotting factors at the site of bleeding where subsequent clot forms.[10] Willna and co-workers also find that gastroprotective benefits of clinoptilolite may be due to its binding to hydrogen ions and biologically active amines/nitrates.[9] However, limited progress has been made in the fundamental understanding of interactions between functional proteins (enzymes) or cells with hemostatic materials in this biological process.

Plasma proteins adsorption to nanomaterial surface have great influence on their bio-functionality.[11, 12] In our previous work, we have discovered that the in-situ generated thrombin in the protein corona of calcium (II) exchanged zeolite surface displays a calcium (II)-dependent, unusually high (~3000 NIH U/mg) procoagulant activity, which is even stable against anti-thrombin deactivation.[13] Our observations suggest that the thrombin activity can be regulated by inorganic surface and cations. Most importantly, our discovery indicates the linkage of the biomolecules in the protein corona to the procoagulant activity of the materials, providing a new molecular basis of the procoagulant mechanism for zeolite hemostatics. These results encourage us to further explore their medical applications in gastrointestinal disorders.

Table 1 Molecular formula and Si/Al ratio of Na-Y and Ca-Y zeolite.

Zeolite	Molecular formula	Si/Al[a]
Na-Y	$Na_{0.55}(SiO_2)_{1.03}(AlO_2)_{0.40}\bullet\chi H_2O$	2.56
Ca-Y	$Ca_{0.17}Na_{0.12}(SiO_2)_{1.08}(AlO_2)_{0.42}\bullet\chi H_2O$	2.57

[a]Si/Al: Atomic ratio of Si to Al.

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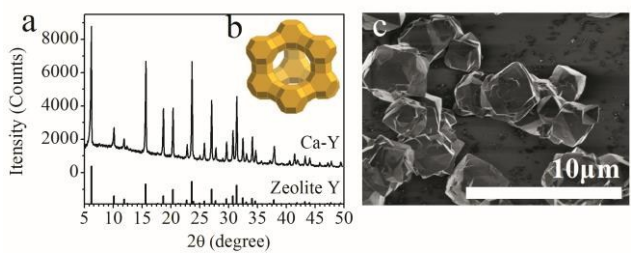

Fig. 1 (a) Powder X-ray diffraction (XRD) pattern of Ca-Y (upper) and standard pattern of zeolite Y (lower). (b) A schematic of zeolite FAU structure where each vertex represents alternating Si and Al atoms and the straight line represents bridging oxygen atoms. (C) Scanning electronic microscopy (SEM) of Ca-Y.

In this work, we explored the *in vitro* procoagulant activity of calcium (II) exchanged zeolite Y (Ca-Y) and its composite with hard protein corona (Ca-Y/HPC), which was pre-treated with artificial gastric juice. Aside this, we studied *in vivo* potency, specifically the gastroprotective effect, by using an ethanol-HCl induced gastric ulcer in a mice model.

The Ca-Y was prepared from commercially available sodium zeolite Y (Na-Y) by standard ion exchange method.[13] The Si/Al ratio and an ion exchange degree by calcium(II) is determined to be 2.57 and 74%, respectively (Table S1, ESI†). SEM images suggest that Ca-Y has a crystalized particle morphology, with diameter distribution in 2.48 ± 0.93 μm (Fig. 1c, Fig. S1, S2, ESI†). The XRD pattern of Ca-Y confirms that the zeolite sample exhibits the FAU structure (Fig. 1a). The unit cell is cubic (a = 24.7 Å) with Fd-3m symmetry and it has a 3-dimensional pore structure with pores running perpendicular to each other in the x, y, and z planes (Fig. 1b). The pore diameter is large (7.4 Å) since the aperture is defined by a 12 member oxygen ring, and leads into a larger cavity o diameter 12 Å.

Ca-Y/HPC was prepared by mixing the Ca-Y and porcine plasma, which is identical to our previous report.[13] The procoagulant activity was evaluated using an *in vitro* clotting assay (ESI†). It measures the coagulation response in terms of clotting time (CT), defined as the time required from activation of the intrinsic pathway of coagulation cascade to the appearance of a firm clot (Fig. S3, ESI†). In this work, the hemostatic agents were evaluated in a mimic gastric environment by pre-treated with artificial gastric juice or deionized water as reference. A thrombin molecule was used as a control sample since it is a vital clotting factor, and has shown effectiveness to control haemorrhage from gastric varices.[14, 15]

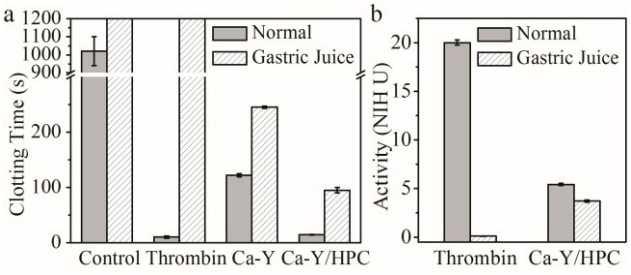

Fig. 2 (a) Procoagulant activity by *in vitro* clotting assay. (b) Thrombin enzyme activity by thrombin chromogenic assay. Assays were conducted under two different conditions, normal or pre-treatment with gastric juice.

As showed in Fig. 2a, the porcine plasma without addition of any agent clots slowly in approximately 17 minutes in normal conditions. The introduction of Ca-Y significantly accelerates the coagulation process, resulting in a short CT of 122 ± 3.0 s. Thrombin (20 NIH U), and Ca-Y/HPC both perform excellent procoagulant activity with CT of 10.5 ± 2.0 s and 14.5 ± 0.5 s, respectively. However, after treatment with artificial gastric juice, plasma with/without addition of thrombin molecules fails to clot within 20 minutes. The thrombin activity assay confirms that the thrombin molecules completely lost their activity after the treatment of artificial gastric juice, implying that it has been denatured and cannot maintain their basic bio-function under such acidic condition (Fig.2b). On the contrary, Ca-Y and Ca-Y/HPC still exhibit effective procoagulant activity with CT of 245 ± 2.0 s and 95 ± 5.0 s, respectively (Fig.2a).

The moderate procoagulant activity of Ca-Y indicates the stability of inorganic materials against harsh biological environment. And further, the significantly reduced clotting time (from 245 s to 95 s) of Ca-Y/HPC can be attributed to the stable in-situ generated thrombin in hard protein corona. Approximately, 68% thrombin activity of Ca-Y/HPC remains after treatment of gastric juice as determined by the thrombin chromogenic assay, in stark contrast to the free thrombin molecules (Fig.2b). The remained activity of thrombin in Ca-Y/HPC is very important due to its crucial role in physiological and pathological coagulation as well as wound healing. The *in vitro* clotting assay results imply that zeolite based hemostatic agents is a promising option to control hemorrhage and associated diseases in the gastrointestinal tract.

The gastroprotective effect of Ca-Y was further evaluated in an ethanol-HCl induced gastric ulcer in a mice model. Gastric ulcer lesions were induced by oral administration of 0.2 mL HCl/ethanol aqueous solution (200 mM HCl in 50% ethanol). Half hour before ulcer induction, the Ca-Y suspension was administered at oral doses of 0.5 g/kg, 2.5 g/kg, and 5.0 g/kg. In this work, the thrombin (2500 NIH U/kg) and Omeprazole (4.5 mg/kg) treated groups are included as references, since Omeprazole is a proton-pump inhibitor medicine, which has been widely used in gastric disorders by suppressing gastric acid secretion. One hour after ulcer induction, intense gastric mucosal damage in the form of hemorrhagic streaks and tissue necrosis were formed (Fig. 3a), which is consistent with previous report.[16] After dissection, intragastric pH was recorded, and gastric ulcer area percentage were measured, which was used as an indicator of the degree of ulcer.

Compared with the control group, oral administration of Ca-Y (2.5 g/kg) significantly reduced gastric ulcer area percentage from 35.1 ± 4.4% to 17.8 ± 2.0%, and improved the intragastric pH from 2.0 ± 0.5 to 3.5 ± 0.5, while the administration of thrombin (2500 NIH U/kg) did not show a statistical discrepancy with 32.9 ± 3.3 % ulcer area percentage and 2.0 ± 0.5 intragastric pH (Fig. 3g). Combined with the *in vitro* tests above, it is reasonable to observe the ineffectiveness of soluble thrombin molecules since it is deactivated in gastric environment.

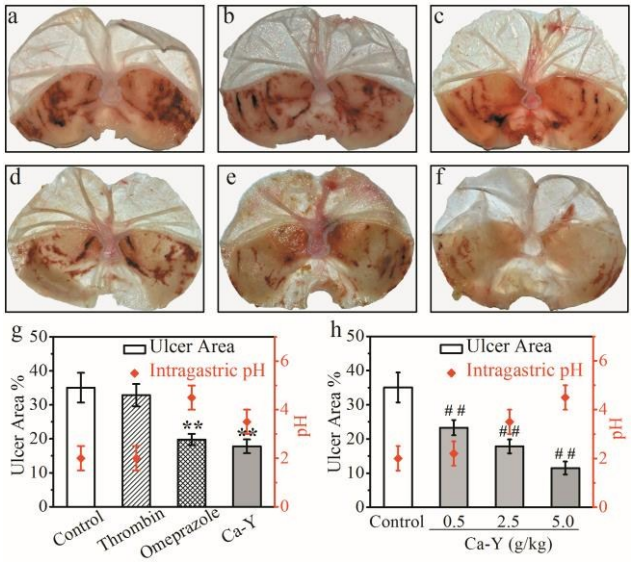

Fig. 3 Gastroprotective effect of Ca-Y on gastric ulcer model induced by ethanol-HCl in mice. The optical images of the stomachs from the mice with pre-oral administration of (a) saline, (b) thrombin (2500 NIH U/kg), (c) Omeprazole (4.5 mg/kg), Ca-Y (d) (0.5 g/kg), (e) (2.5 g/kg), and (f) (5.0 g/kg). Statistical histogram of ulcer area percentage (g) in different groups, * $p < 0.05$, ** $p < 0.01$ vs. control mice, Student's t-test; and (h) in different dosage of Ca-Y, # $p < 0.05$, ## $p < 0.01$ vs. control mice, one-way analysis of variance (ANOVA). n = 6 for each group. Each data was presented as the means ± standard error (SEM). Intragastric pH were also showed.

The Ca-Y shows high gastroprotective potency. As shown in Figure 3h, the oral treatment of Ca-Y by 0.5 g/kg, 2.5 g/kg and 5.0 g/kg can significantly reduce the ulcer area percentage. Meanwhile it displays a dose-dependent manner in both ulcer area reduction and intragastric pH rise (Fig. 3h). This result is in good agreement with previous report that zeolite is an effective antacid for gastric hyperacidity by proton exchange and hydrolysis of presented species.[17]

It is worthy of noting that oral ingestion of Ca-Y in a dosage of 5.0 g/kg can effectively increase intragastric pH as high as 4.5 ± 0.5, which approximately same with Omeprazole in dosage of 4.5 mg/kg. However, the ulcer area percentage treated by the Ca-Y is much less than Omepazole group at this dosage level (11.5 ± 1.9 % vs 19.8 ± 1.7 %). It implies that, in addition to anti-acid effect, there exist other beneficial factors from Ca-Y participating in treating gastric ulcer. In our *in vitro* procoagulant activity test, we found that the in-situ generated thrombin in Ca-Y/HPC surface also showed a good resistance to acidity and pepsin, which can be formed rapidly upon contact with the bleeding site or wound. We thus expect that the stable thrombin function in HPC formed on Ca-Y surface provides a positive influence towards wound healing and tissue repair process[18] in gastrointestinal injury. Eventually, this synergetic effect enables Ca-Y zeolite good gastroprotective function and performs well in *in vivo* experiments. This may provide an optional therapy for gastrointestinal disorders, and more importantly a different view of bio-functionality of inorganic materials.

Conclusions

In this study, the *in vitro* procoagulant activity test reveals the unusually good resistance of Ca-Y zeolite to gastric juice, and later, the *in vivo* experiment of ethanol-HCl induced gastric ulcer in a mice model shows that an oral dosage of 5.0 g/kg can significantly reduce ulcer area percentage from 35.1 ± 4.4 % to 11.5 ± 1.9 %, and improve intragastric pH from 2.0 ± 0.5 to 4.5 ± 0.5 respectively. This gastroprotective effect was considered to be contributed synergistically by 1) the excellent hemostatic ability and consequently formed stable highly active thrombin on the surface of Ca-Y, and 2) efficient anti-acid property, which can improve intragastric pH. It reveals the linkage of zeolite mediated thrombin activity to its therapeutic action in gastric disorders, providing a novel aspect for investigating the biological behaviours of inorganic materials in harsh physiological environment, which is crucial to their biological and medical applications.

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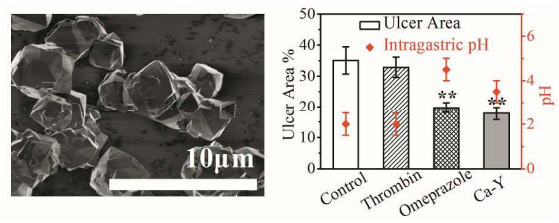

A calcium exchanged zeolite with high hemostatic ability in therapy of gastric ulcer in a mice model.