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Research Article

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Ribonuclease A-Mineral Carrier Complex as a Potential Antitumor Preparation

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Abstract

Objective: The oral administration of therapeutic proteins is complicated by their instability, which leads to loss of their activity in the gastrointestinal tract. Methods of protecting proteins from proteolysis are usually based on their encapsulation, which delays protein release in the stomach and intestines. Nevertheless, the protein is released immediately after capsule damage. The use of sorbents is particularly promising because they provide prolonged protein release. The aim of this work was to create an organo-mineral preparation of antitumor ribonuclease (RNase) immobilised on hydrated aluminosilicate clinoptilolite, which is the safest agent with detoxifying, antioxidant, and anti-inflammatory properties among the numerous zeolites.

Materials and Methods: We developed a new method for processing natural clinoptilolite samples from Tatarsko-Shatrashanskoe deposit for use as sorbents for antitumor RNase A. Zeolites' sorption capacity was analysed, their elemental composition was determined using scanning electron microscopy, images were created using transmission electron microscopy, and porosity was examined using X-ray computed tomography.

Results: We obtained a detailed characteristic of the mineral carrier and selected the optimal zeolite particle size and conditions for RNase A loading/unloading. The dynamics of RNase A release from clinoptilolite showed that the enzyme was gradually released over 20 h while maintaining its stability and catalytic activity in gastrointestinal fluids.

Conclusion: The combination of clinoptilolite detoxifying activity and antitumor activity of RNase A allows us to consider the obtained preparation not only as a factor of body nonspecific defence, but also as a promising antitumor drug supplementing well-known chemotherapeutic agents used in the treatment of neoplasms in the gastrointestinal tract.

Keywords Zeolite · Porosity · Sorption · Ribonuclease A · Protein release · Enzyme stability

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INTRODUCTION

Therapeutic proteins, including vaccines, sera, immunoglobulins, insulins, interferons, *etc*., have been used since the 1950s. In 2017, there were more than 239 FDA-approved peptide drugs available in 380 dosage forms on the US market, about 140 therapeutic peptides were undergoing clinical trials, and more than 500 therapeutic peptides were at the preclinical development stage.[1] The modern strategy of cancer therapy is increasingly turning to the use of monoclonal antibodies, which selectively recognise tumour cells expressing certain oncogenes and slow down or stop their growth.[2,3] Given that protein preparations, when administered orally or intravenously, undergo proteolysis, it is necessary to develop approaches to maintain their initial properties to combat various types of diseases. Our previous studies have established that mineral carriers of therapeutic proteins not only successfully bind target proteins, but also prolong their release.[4,5] In this work, we turned to known mineral carriers–zeolites, which are aqueous calcium and sodium aluminosilicates from a subclass of framework silicates, which proved to be good candidates as adsorbents for anionic [4] and cationic[5] proteins.

Inside the aluminosilicate frame are metal cations such as Ca^{2+}, K^{+}, Na^{+}, Mg^{2+} *etc*., which balance the negative charge of the frame with weak electrostatic bonds, enabling ion-exchange reactions. However, the molecular weight of proteins and their 3D conformation do not allow them to penetrate into micropores of 2-15 Å in size, which make up the primary porosity of the zeolite "windows" due to its crystalline structure. Proteins can penetrate the system of secondary porosity – mesopores and macropores between zeolite particles, which do not have the property of selective adsorption. According to mineral type, most of the deposits, including the Tatarsko-Shatrashanskoye deposit of zeolite-containing marl-siliceous sedimentary rocks, contain clinoptilolite. It forms three-dimensional framework structures consisting of SiO_4 and AlO_4 tetrahedra. Cations compensate the excess negative charge on the anionic part of the aluminosilicate skeleton of the zeolite. Spherical clinoptilolite is the least toxic compared to fibrous and lamellar zeolites,[5-7] and therefore, it is widely used as a sorbent in water and soil purification and in various detoxification processes. The world's largest producers of zeolite are Zhengzhou Fulong New Material Technology Co. (China), Clariant company (Germany), Zeodetox (Serbia), Zeol–"Volga Region Zeolites" (Tatarstan, Russia). Interest in the use of clinoptilolite in veterinary and medicine is constantly growing.[8,9] The mesoporous structure of clinoptilolite-type of natural zeolite particles provides a unique platform for preparation of multifunctional magnetic, optical, and dye-containing probes suitable for optical imaging, magnetic resonance imaging, thermo- and phototherapy and as effective containers for controlled drug delivery.[10,11] Silver-coated zeolite could be used as a filter material in air cleaners to remove bio-aerosols in the respiratory care ward in hospitals and maintain the indoor air quality.[12] Biosensor applications of bioactive zeolitic imidazolate frameworks have a great interest for integrating bioactive molecules such as DNA, aptamers, and antibodies.[13] Young rats displayed kidney dysfunction and hypertension, which was prevented by NO donor (S-nitrosoglutathione) loaded on Cu^{2+}-doped zeolitic imidazolate framework nanoparticles. These results cast new light on targeting NO delivery by using nanoparticles to improve child-focused outcomes related to chronic kidney disease.[14]

Here, we set a goal to characterise the sorption capacity of a natural zeolite, namely, clinoptiolite from the Tatarsko-Shatrashansky deposit in the central European part of the Russian Federation, in relation to the well-known cationic protein, pancreatic ribonuclease A (RNase A), possessing antitumor activity. Bovine pancreatic RNase A belongs to a unique class of cytotoxic and non-mutagenic enzymes that have attracted attention as potential anticancer agents. It is believed that cellular RNase inhibitors (RI) limit the clinical use of the enzyme.[15] However, we chose this enzyme for sorption on zeolites based on the following facts: (i) it was found that after penetrating RNase A into cells of human epidermoid cervical carcinoma HeLa and mouse melanoma B16, part of the enzyme remains unbound to RI and reduces the level of RNA in these cells, leading to inhibition of protein synthesis and induction of apoptosis;[16] (ii) the exclusive role of the enzymatic activity of RNases in cytotoxicity is controversial – data show that the structural organisation of catalytically inactive enzymes contributes to cytotoxicity, determining their interaction with cell components;[17] (iii) complex therapeutic systems based on the use of RNase A-associated nanoparticles have been developed, which induced apoptosis of SW-480 cells of primary intestinal adenocarcinoma.[18]

The existing strategies for protein encapsulation/conjugation and recent advances in the development of protein delivery vehicles include lipid-based membrane nanocarriers, polymeric carriers, metal-organic frameworks, inorganic carriers, protein/peptide-based nanocarriers, and DNA nanostructures.[19] Inorganic materials such as microporous zeolites and mesoporous silicon, as well as synthetic zeolitic imidazolate frameworks, can achieve controlled drug release because of their definite relative porosity. Here, we used for

the first time a natural clinoptilolite-containing rock for the sorption of an enzyme with antitumor activity. The advantage of such complexes, in contrast to synthetic frameworks, is their cost-effectiveness. To confirm the functional activity of the complex, we experimentally validated the parameters of RNase A sorption on the carrier, taking into account its main characteristics, recorded the dynamics of the enzyme release from the carrier, and verified that RNase A retains catalytic activity in simulated gastrointestinal fluids.

Considering the aforementioned facts, the objectives of the experimental work included determining the optimal size of zeolite granules, zeolite processing parameters, and porosity to obtain the required protein loading as well as recording the dynamics of enzyme release and its stability in the fluids of the gastrointestinal tract. Thus, we substantiated the development of a new organomineral drug as a potential therapeutic agent that complements known antitumor agents for combating stomach and intestine neoplasms.

MATERIALS AND METHODS

Enzyme

RNase A (RPA Vector, Russia) from bovine pancreas (EC 4.6.1.18) was used. This is a small cationic protein consisting of 124 amino acids with a molecular weight of 13.7 kDa. RNase A has an activity of 9.7 ± 0.12 × 10^5 units/mg as determined by the hydrolysis of high-polymer yeast RNA using the modified Anfinsen method.[20]

Zeolite

We used clinoptilolite taken from a depth of 20 m in the Tatarsko-Shatrashanskoye deposit. The clinoptilolite in the granular sample had a size of 0.2–0.8 mm and was finely dispersed – less than 40 μm. The samples were subjected to high-temperature treatment for 30 min at a temperature of 500°C (EKPS 50 muffle furnace, model 5007, Russia). Additionally, the zeolite samples were washed twice with 96% ethanol and dried at room temperature.

Images and Spectral Analysis of Zeolites

Both granular and finely dispersed (or powder) samples were washed with 96% ethanol and sonicated to prevent particle aggregation (10 min, 35 kHz, 130 V, Sapphire, Russia). A drop of each sample was placed on a carbon-coated grid and was, after ethanol evaporation, analysed using a transmission electron microscope (HT7700 Exalens (Hitachi High-Tech Science Corporation, Japan) at a resolution of 1.4 Å. Bright-field images were obtained at an accelerating voltage of 100 kV using an AMT XR-81 camera. The elemental compositions were determined using scanning electron microscopy (Carl Zeiss Merlin with an AZtec X-MAX energy dispersive spectrometer, Germany). Elemental analysis was performed at an accelerating voltage of 20 keV and a working interval of 9 mm. A set of reference standards for X-ray microanalysis was used in the AZtec program.

Sample Porosity Analysis

The study of the pore structure of the zeolite samples was carried out using a computed tomography scanner with a nanofocus tube of a Phoenix V|tome|x S240. To create a series of images, the sample was placed in a rotating holder. The images were formed on a digital silicon matrix installed opposite to the X-ray gun as pixel images that were converted into a three-dimensional model, where the brightness characterises the degree of X-ray absorption as a result of the photoelectric effect and Compton scattering.

Loading and Release of RNase A from Zeolite

Zeolite samples (50 mg each) were preliminarily washed thrice with MQ water and dried at 160°C for 30 min. For loading, each sample of zeolites was suspended in 10 mL of water solution of RNase A (concentration, 0.5–1 mg/mL, catalytic activity, 9.7 ± 0.12 × 10^5 U/mg), homogenised on a vortex, and subjected to ultrasonication on ice (5 min, 35 kHz, 120 V), after which it was stirred on a shaker for 2 h and precipitated at 4300 g for 5 min. To determine the zeolite loading capacity, we measured the residual amount of protein in the resulting supernatants by absorption at 280 nm. The values of these indicators for the initial solution of the enzyme were taken as 100%. The supernatant was separated from the loading solution. The precipitates were dried at 50°C for 30 min, then 10 mg of the samples were resuspended in 10 mL of MQ-water and incubated at room temperature, taking 0.5 mL of the supernatant every hour to determine the amount of released protein.

Model Fluids of the Gastrointestinal Tract

A buffer simulating the contents of the large intestine was prepared in accordance with the following recipe (g/L): KCl: 0.2; NaCl: 8; KH_2PO_4: 0.24; Na_2HPO_4: 1.44; pH 7.0.[21] The simulated gastric juice contained (g/L): peptone: 8.3; glucose: 3.5; NaCl: 2.05; KH_2PO_4: 0.6: $CaCl_2$: 0.11; KCl: 0.37; bile: 0.05; lysozyme: 0.1; with 1N HCl.[22] RNase A was dissolved in the prepared solutions and incubated for 20 h at 37°C with periodic measurement of ribonucleolytic activity.

Statistical Analysis

Statistical analysis and approximation of dependencies were performed using OriginPro 2015 (OriginLab Corp., Northampton, MA, USA). The average of three measurements

and the standard deviation were determined. The statistical significance level was taken as $p \leq 0.05$.

RESULTS

Effect of High-Temperature Treatment on Zeolite

The natural zeolite of the Tatarsko-Shatrashanskoye deposit belongs to the sedimentary type, which means that it contains residual organic substances originating from previously living organisms.[23] Therefore, we first analysed the yield of residual organics from unloaded samples of used zeolites at 280 nm to assess the presence of amino acids, mainly phenylalanine, tryptophan, and tyrosine, and at 260 nm to identify the bases of adenine and uracil as components of DNA and RNA.[24,25] It was established that the yield of compounds absorbing in this range from unloaded zeolite-containing rocks did not exceed 0.35 units after 3 h of incubation of the samples in water for untreated zeolite and 0.15 units for heat-treated ones, indicating the burnout of bioorganics during heat treatment of the rock (Figure 1a and Figure 1b).

It was established that organic compounds gradually leave the samples of both granular and powder zeolite, and the maximum yield was observed after 2 h of incubation. Then, the dynamic balance of the suspension led to partial reabsorption

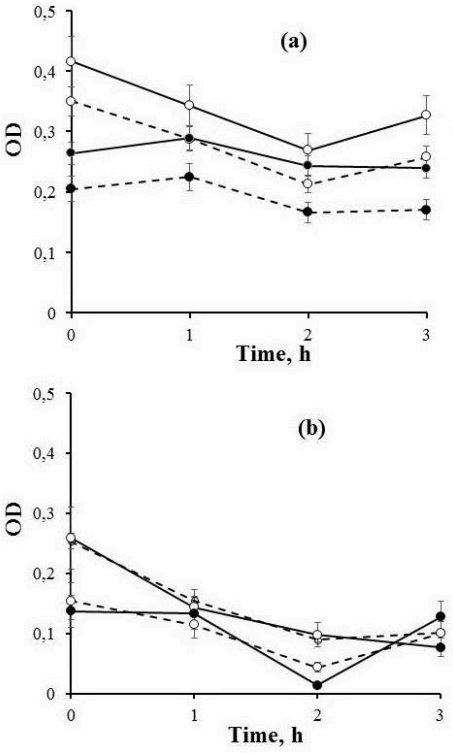


Figure 1. Release of residual organic matter from powder (hollow markers) and granular (solid markers) zeolites, measured by supernatant absorbance at 260 nm (solid line) and 280 nm (dashed line) after zeolite incubation in distilled water. (a) untreated zeolite sample; (b) zeolite treated for 1 h at 500°C.

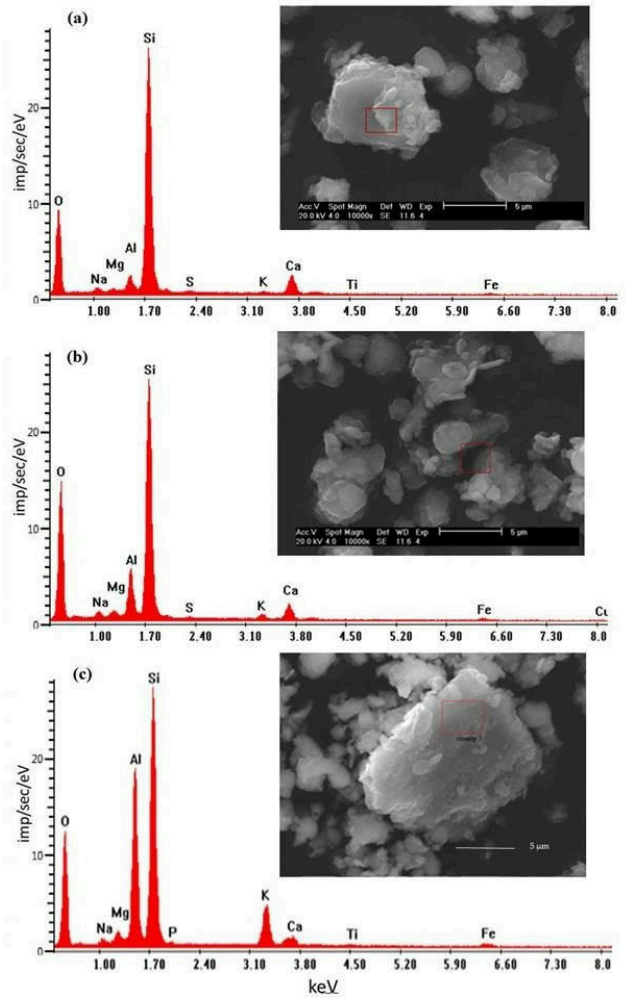


Figure 2. Elemental composition and microphotographs of the zeolite samples: (a) granular sample treated at 500°C; (b) powder sample treated at 500°C; (c) original untreated rock. The square in the photo is the localisation of spectral analysis points.

of organic compounds by the mineral. Thus, high-temperature treatment of zeolite was important for eliminating residual organic matter and subsequent optimisation of therapeutic proteins sorption. The absorption levels at 260 and 280 nm and their dynamics did not fundamentally differ. Therefore, in the future, we will conduct measurements at 280 nm, which is characteristic of the protein.

At high temperatures, aluminium atoms are removed from the zeolite lattice via a process called dealumination. This was due to dehydroxylation (release of water during the interaction of two OH groups), accompanied by the release of part of the aluminium atoms from tetrahedral positions with the formation of aluminium-oxygen fragments in the cavities of the zeolite.[26,27] The heat treatment of the samples decreased aluminium as well as potassium content compared with the original rock (Figure 2).

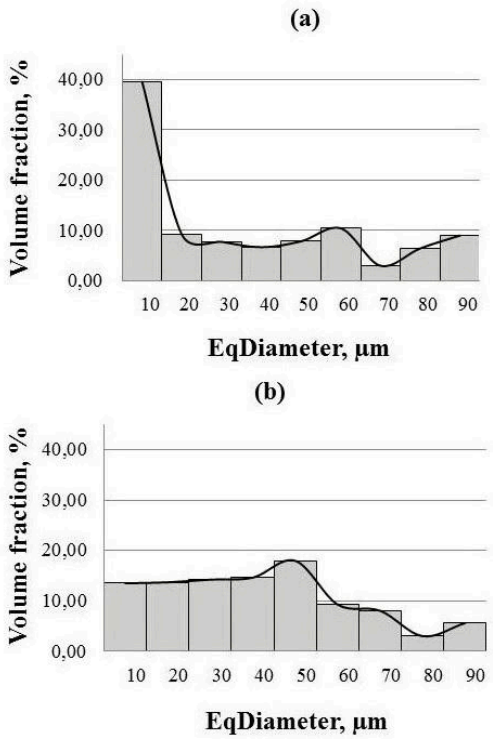


Figure 3. Pore distribution diagram according to equivalent diameter. (a) untreated sample; (b) ethanol-treated sample.

The partial elimination of organics during high-temperature treatment leads to an increase in the sorption capacity of zeolites. In this case, the carrier itself became practically sterile, which is important for its subsequent loading with therapeutic proteins.

Effect of Ethanol Treatment on Zeolite

Not only calcination but also washing with ethanol showed a positive effect, expressed in a decrease in the proportion of superfine pores and an increase in the proportion of pores with an average size in the samples (Figure 3).

Washing out nanosized particles redistributed the pore size. The small pores that predominate in the untreated sample coalesced into larger pores suitable for incorporating protein molecules (Figure 4). Unfortunately, X-ray computed tomography did not allow us to obtain the same sample before and after alcohol treatment. Despite the fact that the orthogonal section gives only a limited partial picture of the reduction in the number of small pores in the sample, the tomographic image processing program allowed us to isolate the entire volume of pores in the sample and then separate them according to the required parameters, but this fact was confirmed by the pore distribution diagram in Figure 3.

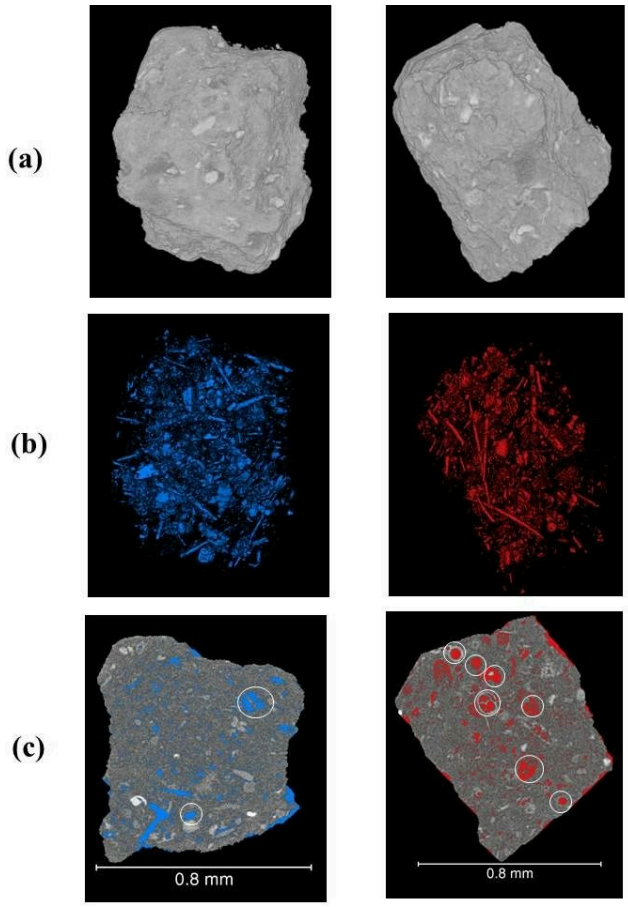


Figure 4. X-ray computed tomography of zeolite-containing rock samples from Tatarsko-Shatrashanskoye deposit. (a) form of the samples; (b) 3D visualisation; (c) orthogonal slice with pores highlighted in blue and red. Left column: untreated sample; right column: ethanol-treated sample.

RNase A Loading and Unloading

Comparison of the zeolite adsorption capacities showed that finely dispersed zeolite adsorbed RNase A almost immediately within 2 h. Granular zeolite adsorbed more protein over time (up to 3 h) than finely dispersed (Table 1). Moreover, granular zeolite gradually adsorbed RNase A, which suggests its stronger future retention in zeolite and its gradual release. As shown in Figure 1, increasing the incubation time of RNase A with zeolite was inappropriate because it led to partial readsorption of the protein. We hypothesised that a decrease in the concentration of RNase A in the initial solution could slow down the loading rate and increase its level. However, powder zeolite adsorbed the maximum amount of protein after 0.5 h because the loading level did not increase (Table 2) with time (Table 1).

Table 1. Comparison of RNase A loading in the powder and granular zeolite fractions.

	Granular zeolite fraction				Powder zeolite fraction			
Time, h	0	1	2	3	0	1	2	3
Loading solution, OD_{280}	1.5	1.0	0.72	0.55	1.4	0.58	0.47	0.60
Enzyme content in zeolite, %[a]	0	33.3	52.0	63.3	0	58.6	66.4	57.1

[a] Initial content of RNase A in the solution (1000 μg/ml, D_{280} = 1.45 ± 0.05) was taken at 100%. The standard deviations of the three experiments did not exceed 10% (σ ≤ 10%).

The fact that powdered zeolite releases all adsorbed proteins within 2 h (Table 2) makes it almost unsuitable for use as a carrier that should release the therapeutic protein gradually.

Based on the obtained data, we opted for granular zeolite as a more preferred carrier for RNase A. Indeed, the loading of RNase A into this zeolite sample occurred slowly within 3 h. The granular zeolite adsorbed approximately 72% of the enzyme (Table 3). When we measured protein release in aqueous solution without changing the solution, the achieved dynamic equilibrium limited protein release. However, oral drugs move along the gastrointestinal tract, allowing the therapeutic substance to be released according to the concentration gradient.

We carried out a model experiment by centrifuging the aqueous solution every hour where the enzyme was released and replacing it with fresh water. Thus, we achieved gradual release of the enzyme, with approximately 42% of RNase A released within 4 h. The enzyme was almost completely released within 20 h (Table 3).

Preservation of RNase A Catalytic Activity in Gastrointestinal Fluids

The total time taken for food to transit through the gastrointestinal tract is 36-48 h. Food was stored in the stomach for about 0.5-2 h, in the small intestine 1-4 h, and in the large intestine 30-46 h.

Thus, the loaded zeolite, after a supposed single oral ingestion, can provide a release of RNase A throughout the gastrointestinal tract, which, in the presence of tumours, can exert its therapeutic effect. However, the prospects for oral administration of RNase A on a carrier should need confirmation by the preservation of enzymatic activity in the gastrointestinal tract. We found RNase A to be stable in the colonic environment. There were no statistically significant differences in the values of the catalytic activity compared with the activity in aqueous solution (Figure 5).

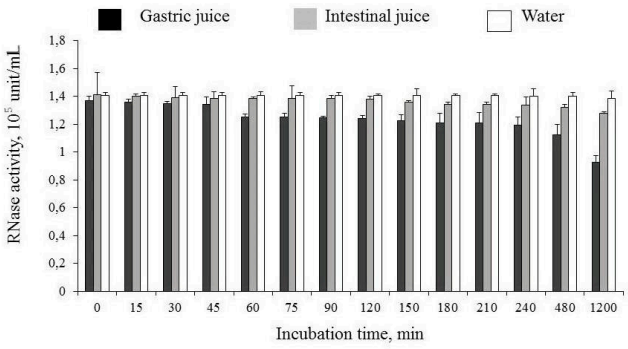


Figure 5. Dynamics of RNase A activity preservation in gastrointestinal tract model fluids during 20 h of incubation.

At the same time, RNase A was less stable in gastric juice–in experiments with the main dose (100 μg/mL), and the loss in

Table 2. Evaluation of RNase A loading into powder zeolite and its unloading without changing the aqueous solution into which the enzyme was released.

RNase A loading[a]					RNase A unloading[b]				
Time, h	0	0.5	2	3	Time, h	0	2	3	4
Loading solution, OD_{280}	0.498	0.214	0.216	0.215	Unloading solution, OD_{280}	0.07	0.201	0.213	0.214
Enzyme content in zeolite, %	0	57.02	56.62	56.82	Enzyme release rate, %	32.56	93.49	99.07	99.53

[a] for loading, the initial content of RNase A in the solution (500 μg/ml, D280 = 0.498) was taken as 100%; [b] for unloading, the content of RNase A in the loaded zeolites was taken as 100%. The standard deviations of the three experiments did not exceed 12% (σ ≤ 12%).

Table 3. Evaluation of RNase A loading into granular zeolite with gentle stirring and its unloading with an hourly change of the aqueous solution into which the enzyme is released.

RNase A loading[a]					RNase A unloading[b]					
Time, h	0.1	1	2	3	Time, h	0.1	2	3	4	20
Loading solution, OD_{280}	1.5	1.0	0.7	0.5	Unloading solution, OD_{280} Σ(t) D_{280}	0.173 0.173	0.086 0.259	0.093 0.352	0.196 0.548	0.432 0.980
Enzyme content in zeolite, %	16.7	44.4	61.1	72.2	Enzyme release rate, %	13.3	19.9	27.1	42.2	94.5

[a] for loading, the initial content of RNase A in the solution (1000 μg/ml, D_{280} = 1.45 ± 0.05) was taken as 100%; [b] for unloading, the content of RNase A in the zeolite was taken as 100%. The standard deviations of the three experiments did not exceed 12% (σ ≤ 12%).

activity averaged 11.6 ± 0.52% ($p \leq 0.05$) after 4 h of incubation, and a third of the activity was lost only after 20 h. This result indicated that RNase A released from carriers retained its catalytic activity during transit through the intestine.

DISCUSSION

The importance of zeolites as detoxifying and ion-exchange agents is already evident in the agroindustrial and zootechnical fields. In veterinary medicine, zeolite improves the fitness of pets and removes radioactive elements, aflatoxines, and poisons.[28] Zeolites are also successfully used as carriers of various useful agents, in particular, siderophore, to fight iron deficiency-induced chlorosis, which is a widespread agricultural problem that can lead to massive crop failures.[29] Zeolite also displays antioxidant, whitening, haemostatic and anti-diarrhoeic properties, as projected in human care.[28] Currently, data on the medical use of zeolites in clinical practice are limited. The pilot results of phase I/IIa trials confirm that the oral administration of clinoptilolite may improve the lipid profile of individuals with dyslipidemia.[30] Microporous Faujasite zeolite (NaX-FAU) can be used as a drug delivery system to facilitate the oral delivery of poorly water-soluble compounds.[31] Moreover, zeolite-containing biologically active additives are already being produced as commercial preparations. Clinoptilolite Absorbatox® (an internationally patented non-systemic medical device, ABX PHARMA) is a gastroprotective agent that can reduce the severity of clinical symptoms and signs associated with endoscopically negative gastroesophageal reflux disease and nonsteroidal anti-inflammatory drug medication.[32] A natural zeolite clinoptilolite with enhanced physicochemical properties is the basis of the dietary supplements Megamin and Lycopenomin, which have demonstrated positive effects in immunocompromised patients.[33] Megamin (Tribo Min, Croatia) is recommended as an additional source of minerals (calcium and magnesium) and for concomitant therapy in patients with cancer. Megamin leads to elimination of the side effects of chemo- and radiation therapy (hair loss, reduction and prevention of intoxication, nausea and vomiting, prevention of toxic polyneuropathy, suppression of red bone marrow activity); in individuals with contraindications to chemo- and radiation therapy, it induces the correction of immune status, reduction of carcinoid intoxication, normalisation of appetite, and reduction of pain syndrome. Thus, zeolites are proposed as a potential co-adjutant of toxic chemotherapy that appears to be the most relevant clinical use of this mineral. In turn, RNase A can be proposed as a potential component of anticancer therapy, which inhibits signaling pathways having tumour-promoting activity and decreases the invasion potential of tumour cells.[34] Based on these two main principles, in this study we created an organomineral preparation with detoxifying and antitumor potential. Firstly, the natural clinoptilolite-containing tuff from a local deposit in the Tatarstan Republic was characterised in detail. The starting material was found to contain residual organic matter, which could be removed by high-temperature treatment (Figure 1). Thermal treatment is a common method used to modify the physicochemical properties of zeolite-based materials, which alters the number and type of acid sites through dealumination and increases molecular diffusion via mesopore formation.[35] We confirmed that high temperature treatment does indeed reduce the aluminium content of the samples, with a higher reduction observed in the granular zeolite compared to the powder zeolite (Figure 2). This processing method should definitely increase the proportion of mesopores, which are larger than micropores. The additional method for treating zeolite with alcohol that we proposed increased the proportion of large pores in the zeolite (Figure 3 and Figure 4).

It should be noted that the particle size of clinoptilolite-containing tuff is critical for enzyme loading and unloading. The differences in the adsorption capacity of zeolite particles of different sizes may not always be significant. For example, the monolayer adsorption capacities of granular mordenite (14.56 mg/g) and powdered mordenite (15.13 mg/g) has nearly similar values.[36] Although it has been reported that the column adsorption capacity of clinoptilolite powders for some pollutants almost tripled that of clinoptilolite granules (130.6 mg/g versus 45.3 mg/g) due to higher specific surface areas,[37] we obtained approximately the same enzyme-loaded amount values after 3 h of loading, namely 66.3% for the granular sample and 57.1% for the powdered one (Table 1). However, RNase A was almost completely released (99%) from the powdered zeolite after just 3 h (Table 2), which is clearly not enough to actualise its antitumor properties in the gastrointestinal tract. Therefore, our choice was made in favour of granular samples. Gentle stirring of the zeolite-enzyme suspension resulted in a slight increase in the loading level (72.2%). The enzyme release corresponded to the requirements for its implementation in the gastrointestinal tract: with an hourly change of the aqueous medium where the enzyme was released, we recorded its gradual prolonged release over 20 h, which reached 94.5% of the loaded amount (Table 3).

The results show that the pores in the zeolite samples have equivalent diameter in μm size. The fraction of pores with smaller diameters was successfully removed by alcohol treatment (Figure 3). Previously, we established that the experimental hydrodynamic size of the RNase binase

molecule determined by NMR techniques does not exceed 4.2 nm in diameter.[38] According to the data presented in Figure 4, the pore size is sufficient for accommodating the antitumor enzyme protein. This enzyme was successfully loaded into natural zeolites, but the lack of processing prevented the RNase from being retained by the carriers for >4 h.[5] Here, we obtained RNase A release during 20 h.

Protein therapeutics have been used to treat various diseases, such as cancer, genetic disorders, autoimmunity, and inflammation. Protein therapeutics have demonstrated advantages such as specific pharmaceutical effects, low toxicity, and strong solubility.[39] To avoid several disadvantages in clinical applications, protein structure modifications and different drug delivery systems are used. Recent progress relates to polymer-based systems, lipid-based systems, and inorganic nanoparticles for sustained-release protein delivery. We used the latter method to create a new protein-mineral composition with detoxifying and antitumor potential that provides sustained release of RNase A and stabilises the protein against various external stresses. It should be emphasised that RNase A has never been used for inclusion in a natural mineral carrier. Zeolites are generally biocompatible and nontoxic, making them suitable for pharmaceutical and biomedical applications.[40] The company "ZEOL" has its own raw material base in the form of a private quarry, located within the boundaries of one of the largest in Russia Tatarsko-Shatrashanskoye deposits of zeolite-containing rocks. The exploration reserves of raw materials reached 88.3 million tons, which provides a real prospect for their large-scale use for the needs of industry, agriculture and biomedicine. The main food applications of zeolites are related to their good adsorbent properties, which can be altered and tuned by ion exchange and surface organo-modification, among others, for a specific designed application.[41] RNase A has been repeatedly used to create conjugates with antitumor activity. An antibody-drug conjugate combining RNase A with cetuximab, an anti-epithelial growth factor receptor monoclonal antibody, through a polyethylene glycol linker was designed to induce apoptosis in KRAS-mutant colorectal cancer via a ROS-mediated pathway.[42] The promising therapeutic potential of covalent organic frameworks, photothermal composite systems modified with multi-armed polyethylene glycols, for delivering the protein drug RNase A to treat rheumatoid arthritis was confirmed.[43]

It was shown that the zeolitic-imidazolate framework-8 as an emerging platform has exhibited great potential in protein delivery and was employed as a carrier for the encapsulation and intracellular delivery of RNase A to achieve rapid protein release in an acidic environment. RNase A@ZIF-8 nanoparticles also exhibited excellent biocompatibility.[44] Although three-dimensional zeolitic imidazolate framework-8/sodium alginate-kappa-carrageenan hydrogel beads demonstrated excellent mechanical performance which grant the feasibility of developing cargo delivery in biomedical applications,[45] this framework includes precious metals, making it an expensive and difficult to synthesise. At the same time, clinoptilolite itself demonstrated excellent biocompatibility as a composite scaffold, which improved bone regeneration and promoted repair.[46] Previously, using confocal microscopy, it was shown that RNase A penetrates into HeLa and B16 cells.[47] However, several RNase A superfamily proteins serve as ligands of receptor tyrosine kinases.[48] Note that tyrosine kinases embedded in the cell membrane (EC 2.7.10.1), such as, for example, growth factor receptors, including platelet-derived growth factor receptor and epidermal growth factor receptor, have an extracellular domain, the interaction of RNase A with which is possible even without the enzyme penetrating into the cell leading to blocking of proliferation. In our work, we created a simple system for delivering RNase A to the gastrointestinal tract that will work as an antitumor and detoxifying system when this organomineral complex is taken orally. Undoubtedly, for the further development of our topic, we plan to conduct experiments on animals, confirming the safety of the created organomineral complex and its impact on the intestinal microbiota, and then move on to clinical trials on patients diagnosed with oncological diseases of the gastrointestinal tract.

CONCLUSION

In this study, we designed a new organomineral complex that combines antitumor and detoxifying activities for subsequent use in pharmacology. The processing conditions for carrier clinoptilolite, a mineral of the zeolite group, were selected. High-temperature and ethanol treatment led to dealumination of the clinoptilolite, freeing it from residual organic matter and reducing the number of micropores with diameters less than 10 μm. The clinoptilolite prepared in this way adsorbs more than 70% of RNase A from solution in 3 h. The dynamics of RNase A release from carrier showed that the enzyme is gradually released within 20 h. This time point is sufficient for the gradual release of the antitumor enzyme in the gastrointestinal tract. Of particular importance is the result obtained *in vitro* on model gastric and intestinal fluids, confirming the preservation of the enzyme catalytic activity during 20 h because RNA degradation contributes to RNase A antitumor activity. Complexes of RNase A and clinoptillolite can be synthesised using relatively simple and cost-effective methods, and can retain its functional

activity over a long shelf life, since the commercial RNase A preparation is suitable for 2 years at temperatures up to 25°C,[49] and natural zeolite–clinoptilolite is widely used as a food additive to remove heavy metals, free radicals, toxins, allergens, pesticides, and radionuclides from the body. Thus, the combination of the clinoptilolite detoxifying activity and antitumor activity of RNase A allows us to consider the obtained preparation not only as a factor of body nonspecific defence, but also as a promising antitumor drug supplementing well-known chemotherapeutic agents used in the treatment of neoplasms in the gastrointestinal tract.

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