International Journal of Pharmaceutics 509 (2016) 360–367

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Thiolated graphene oxide as promising mucoadhesive carrier for hydrophobic drugs

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ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form 25 May 2016
Accepted 27 May 2016
Available online 28 May 2016

Keywords:
Mucoadhesion
Graphene
Graphene oxide
Thiolation
Biological barrier
Valsartan

ABSTRACT

The aim of this study was to improve the mucoadhesive properties of graphene by conjugating thiol ligands, in order to formulate an oral delivery system for hydrophobic drugs showing long mucus residence time.

Graphene oxide was obtained by oxidation of graphite and then was thiolated following two synthetic paths. On the one hand, the hydroxyl groups were conjugated with thiourea passing through the formation of a brominated intermediate. On the other hand, the carboxylic acid groups were conjugated with cysteamine via carbodiimide chemistry. The mucoadhesive properties of thiolated graphene were evaluated by rheological measurements and by residence time assay. Then, valsartan was loaded on thiolated graphene and the release profile was evaluated in simulated intestinal fluid.

Following both synthetic paths it was possible to obtain thiolated graphene bearing 215–302 μmol SH/g product. Both products induced after 1 h incubation an increase of mucus viscosity of about 22–33-fold compared to unmodified graphite. The residence time assay confirmed that 60% of thiolated graphene could be retained on intestinal mucosa after 4 h incubation, whereas just 20% of unmodified graphite could be retained. Valsartan could be loaded with a drug loading of about 31 ± 0.3% and a sustained release profile was observed for both formulations.

According to the presented data, the thiolation of graphene could improve its mucoadhesive properties. Therefore, thiolated graphene represents a promising platform for oral delivery of hydrophobic drugs, possessing a long residence time on intestinal mucosa which allows the release of the loaded drug close to the adsorptive epithelium.

1. Introduction

The research interest on graphene has significantly increased in the past years due to its peculiar optical, mechanical, chemical and thermal properties (Geim and Novoselov, 2007; Warner et al., 2013). However, recently this two-dimensional honeycomb structured carbon material started to be relevant also in the pharmaceutical field (Goenka et al., 2014; Wang et al., 2011). Among the graphene-based materials, particular attention was drawn to graphene oxide due to its water solubility, which can be exploited for several biological applications (Miao et al., 2013; Shen et al., 2012; Shim et al., 2014). Other characteristics that make this material so attractive in pharmaceutics are the potential of conjugating molecules via covalent and non-covalent chemistry and its high surface area allowing great drug loading via π stacking and hydrophobic interaction. Covalent conjugations onto graphene oxide have been pursued for the binding of several polymers, as for example PEG, PVA and chitosan, in order to enhance the solubility properties of graphene-based materials (Bao et al., 2011; Salavagione et al., 2009; Shen et al., 2012). Non-covalent chemistry has

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http://dx.doi.org/10.1016/j.ijpharm.2016.05.059
0378-5173/

Nomenclature

EDAC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
G	Ultra fine grinding natural graphite
Gox	Graphene oxide
GSH 1	Thiolated graphene (obtained following synthetic path 1)
GSH 2	Thiolated graphene (obtained following synthetic path 2)
H_2O_2	Hydrogen peroxide 50%
H_2SO_4	Sulfuric acid 98%
$KMnO_4$	Potassium permanganate
LDH	Lactate dehydrogenase
LiBr	Lithium bromide anhydrous
MEM	Minimum essential medium
NBS	*N*-bromosuccinimide
NHS	*N*-hydroxysuccinimide
NMP	*N*-Methyl-2-pyrrolidone
Ph_3P	Triphenylphosphine
TNBS	2,4,6-Trinitrobenzenesulfonic acid

been exploited for the adsorption of low molecular weight heparin in order to generate blood compatible materials as well as for the adsorption of DNA, doxorubicin, camptothecin and folic acid (Lee da et al., 2011; Miao et al., 2013; Wang et al., 2011, 2013).

So far, the potential of graphene-based materials in the pharmaceutical field has been mainly evaluated for gene delivery and cancer therapy, foreseeing a parenteral application rout. With this project we wanted to evaluate the potential of graphene derivatives as oral drug delivery system bearing mucoadhesive properties. The concept of mucoadhesion was developed with the aim to prolong the residence time of the administered formulation at the adsorption site. This provides a steeper concentration gradient close to the epithelium that should improve the permeation of the active ingredient. Many strategies have been pursued in order to create mucoadhesive materials. Among these strategies, one that showed great potential is the thiolation strategy. Thiomers are polymers bearing sulfhydryl ligands that can form disulfide bond with the cysteine-rich regions of the mucus gel layer covering the surface of the gastrointestinal tract (Bonengel and Bernkop-Schnürch, 2014).

Therefore it was the aim of this study to apply the thiolation strategy to graphene in order to improve its mucoadhesive properties. Graphene oxide possess several functional groups that can be exploited for thiolation, namely hydroxyl and carboxylic acid. Moreover the abundance of C=C bound offers also the chance for thiolation (Luong et al., 2015; Orth et al., 2013; Pham et al., 2013; Yun et al., 2015). Within this study graphene oxide was thiolated via substitution of hydroxyl groups with thiol groups passing through the formation of a brominated intermediate and via the conjugation of cysteamine to carboxylic acid groups via carbodiimide chemistry. The amount of conjugated thiol groups was quantified via colorimetric assay, a method well established for polymer characterization and used for the first time with graphene derivative. Then, the mucoadhesive properties of the obtained products were evaluated via rheological assessment when incubated with porcine intestinal mucus and via residence time evaluation in porcine intestinal mucosa. The cytotoxicity of the synthetized products was assessed in Caco-2 cells. Finally, the ability of these materials to adsorb hydrophobic drugs was evaluated. Valsartan was chosen as model active ingredient and the drug loading as well as the release profile in simulated intestinal fluid was assessed.

2. Materials and methods

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), 5,5′-dithiobis(nitrobenzoic acid), 2,4,6-trinitrobenzenesulfonic acid (TNBS), hydrogen peroxide 50% (H_2O_2), lithium bromide anhydrous (LiBr), *N*-bromosuccinimide (NBS), *N*-hydroxysuccinimide (NHS), potassium permanganate ($KMnO_4$), resazurin, sulfuric acid 98% (H_2SO_4), thiourea, triphenylphosphine (Ph_3P), all other salts and solvents at analytical grade were purchased from Sigma–Aldrich (Vienna, Austria). *N*-Methyl-2-pyrrolidone (NMP) and molecular sieves 4 Å were obtained from Carl Roth (Graz, Austria). Ultra fine grinding natural graphite (UF2) was kindly supplied by AMG Mining AG Molecular (Hauzenberg, Germany). Minimum essential medium (MEM) was purchased from Biochrome AG (Berlin, Germany). CytoTox-ONE™ Homogeneous Membrane Integrity Assay Kit (for LDH assay) was purchased from Promega™.

2.2. Synthesis of graphene oxide

The oxidation of graphite was performed following a method previously described (Chen et al., 2013). Briefly, 9 g of graphite (G) were dispersed in 0.21 L of H_2SO_4 in an ice bath under agitation, yielding a black suspension. $KMnO_4$ (21 g) was added slowly under stirring in order to maintain the temperature of the mixture lower than 20 °C. The ice bath was then removed and the reaction mixture stirred for 30 min. Afterwards, 0.45 L of water were added and stirred for 15 min yielding a brown suspension. The mixture was further diluted with 1.5 L of water and mixed for 5 min. Finally 45 mL of H_2O_2 50% were slowly added and the solution turned to yellow. The purification was carried out by filtration. The remained solid was washed with HCl, suspended in 1.8 L of water and dialyzed against water for 7 days. The final product, graphene oxide (Gox), was stirred overnight and then exfoliated by sonication for 30 min. In order to remove un-exfoliated material, the suspension was centrifuged for 40 min at 5000 rpm and the supernatant was finally lyophilized.

2.3. Thiolation of hydroxyl groups

To obtain thiolated G the hydroxyl groups of Gox were first substituted with bromide moieties and then replaced with thiol groups following a synthetic path previously described, adapted to suite the new conditions (Sarti et al., 2010).

Gox was dried at 75 °C under vacuum overnight, LiBr was dried at 90 °C overnight and NMP was stored over molecular sieve 4 Å. All synthetic steps were performed in anhydrous conditions under nitrogen.

Dried Gox (4 g) was dispersed in 300 mL of NMP. Then, 88 g of LiBr were added and stirred for 30 min. Afterwards, 8 g of NBS and 12 g of Ph_3P were added and stirred for 4 h at 80 °C. Finally, the product was diluted with 400 mL of water and purified by filtration. The remained solid was washed with water (c.a. 1 L) in order to eliminate trace of solvent and of unreacted chemicals. The product was suspended in water (c.a. 1 L) and stirred at 10 °C for 1 h in order to improve the purification. After a further filtration and washing with 1 L of water, the product was lyophilized.

The obtained brominated-graphene (GBr) (1.5 g) was dispersed in 200 mL of NMP and then 6 g of thiourea were added and stirred overnight at 70 °C. To the reaction mixture 40 mL of NaOH 3 M were added, stirred for 10 min and then 60 mL of H_2SO_4 3 M were added Finally, the product was filtered and the remained solid purified as